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REVIEW ARTICLE

A liquid biopsy in primary lung cancer

Kazue Yoneda[1] · Naoko Imanishi[1] · Yoshinobu Ichiki[1] · Fumihiro Tanaka[1]

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Abstract
A tissue biopsy is the "golden standard" for molecular profiling that is essential in decision-making regarding treatment for malignant tumors, including primary lung cancer. However, tumor biopsies are associated with several limitations, including invasiveness and difficulty in achieving access. Liquid biopsies have several potential advantages over tissue biopsies, and recent advances in molecular technologies have enabled liquid biopsies to be introduced into daily clinical practice. Cell-free blood-based liquid biopsies to detect mutations in the *epidermal growth factor receptor* (*EGFR*) gene in the plasma have been approved and may be useful in selecting patients for treatment with tyrosine kinase inhibitors of EGFR. We herein describe blood-based liquid biopsies and review the current status and future perspectives of plasma genotyping in primary lung cancer.

Keywords Liquid biopsies · Circulating tumor DNA · Plasma genotyping · EGFR

Introduction

Lung cancer is the leading cause of cancer death worldwide [1] and comprises four major histologic types [small cell lung cancer (SCLC), squamous cell carcinoma (Sq), adenocarcinoma, and large cell carcinoma] [2]. SCLC, accounting for 10–15% of all lung cancers, is a distinct histologic type of neuroendocrine origin characterized by early spread to lymph nodes and distant organs as well as by a higher responsiveness to radiotherapy and cytotoxic chemotherapy than other types [3, 4]. The other histologic types are clinically classified as non-small-cell lung cancer (NSCLC) and uniformly treated as a whole. However, systemic treatment with cytotoxic agents, which is recommended as the standard care of treatment for metastatic NSCLC, may provide only a modest clinical benefit [5].

Recent advances in systemic treatment with agents targeting specific "cancer hallmarks" of individual tumors have drastically changed the treatment strategies for metastatic NSCLC (Fig. 1) [6–9]. Accordingly, the precise identification of molecular characteristics as well as the histologic classification among NSCLC [Sq or others (Non-Sq)] is essential in treatment decision-making, for which tissue biopsies have been traditionally employed [7–10].

For example, mutations in the *epidermal growth factor receptor* (*EGFR*) gene, such as in-frame deletions in exon 19 (Del 19) and a point mutation in exon 21 (L858R), are the most common oncogenic alterations causing NSCLC. These activating *EGFR* mutations are predominantly identified in adenocarcinoma, and the incidence is higher in East Asian patients than in Caucasian patients (approximately 50 vs. 10% for adenocarcinoma) [10–12]. Several randomized clinical trials (RCTs) comparing tyrosine kinase inhibitors (TKIs) of EGFR with traditional cytotoxic chemotherapy including platinum agents for advanced NSCLC harboring activating EGFR mutations (*EGFR*-mutated NSCLC) have been conducted, and all have shown a significantly longer progression-free survival (PFS) in patients treated with an EGFR-TKI [8–10]. Based on these results, systemic first-line treatment with an EGFR-TKI (first-generation agent, gefitinib or erlotinib; second-generation agent, afatinib) is recommended for advanced NSCLC patients with activating mutations (also referred to as "sensitizing mutations"). However, in clinical practice, it is sometimes difficult to obtain adequate tumor tissues that are suitable for molecular characterization, which is particularly crucial in "re-biopsies" to determine the resistance mechanism after the failure of first-line treatment.

✉ Fumihiro Tanaka
ftanaka@med.uoeh-u.ac.jp

[1] Second Department of Surgery (Chest Surgery), University of Occupational and Environmental Health, Japan, Iseigaoka 1-1, Yahata-nishi-ku, Kitakyushu 807-8555, Japan

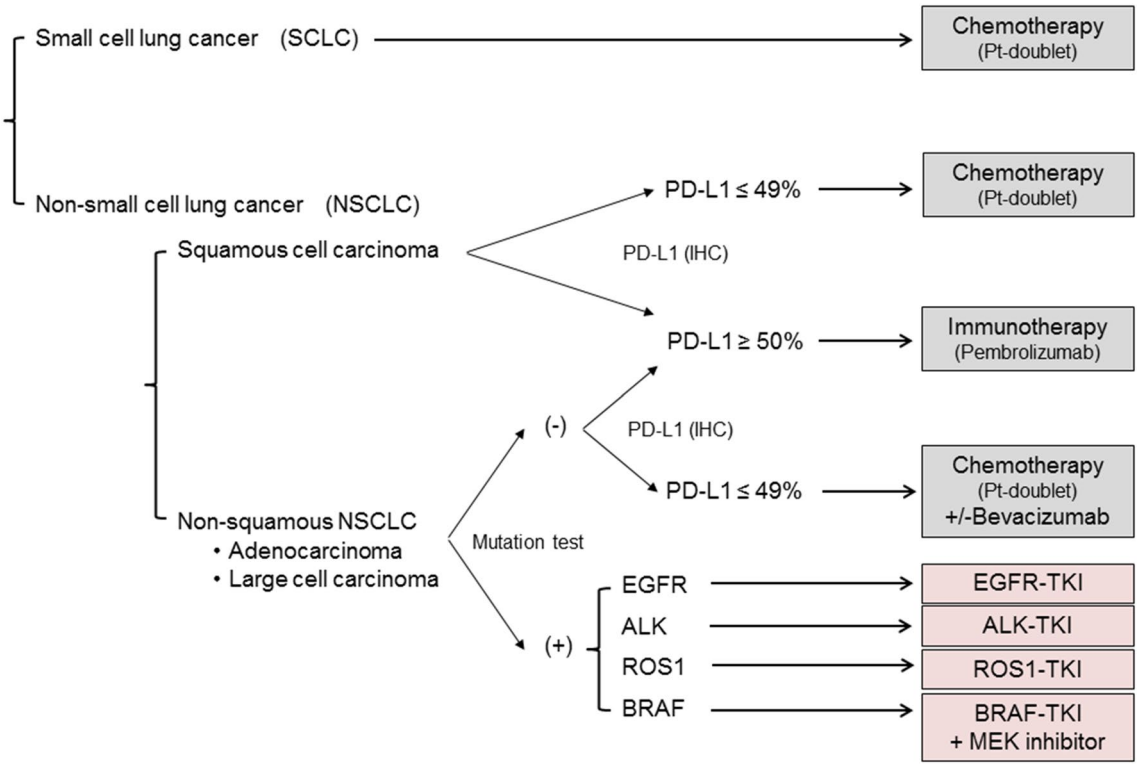

Fig. 1 Treatment strategies for patients with metastatic lung cancer. *Pt-doublet* platinum-agent plus third-generation agent, *PD-L1* programmed death-ligand 1, *IHC* immunohistochemistry, *EGFR* epidermal growth factor receptor, *ALK* anaplastic lymphoma kinase, *TKI* tyrosine kinase inhibitor

Body fluids, such as peripheral blood, urine and saliva, can be easily and repeatedly obtained from patients and may be useful for pathological and molecular characterization through "liquid biopsies" [13] (Fig. 2). Indeed, plasma genotyping for advanced NSCLC patients to detect *EGFR* mutations, including activating mutations to predict the efficacy of EGFR-TKIs and a resistant mutation (T790M) to predict the efficacy of a third-generation EGFR-TKI (osimertinib), has already been approved for clinical use [14]. In addition to their capability to non-invasively detect and monitor gene alterations, liquid biopsies have a number of potential advantages over tissue biopsies (Table 1) [9, 15–19]. For example, they can detect or predict the presence of minimal residual disease (MRD) responsible for tumor recurrence after complete resection, which may be useful for prescribing optimum adjuvant treatment [17, 20].

In the present article, we reviewed the current status and future perspectives of blood-based "liquid biopsies" in NSCLC.

Overview of blood-based liquid biopsies

Tissue biopsies versus liquid biopsies

Tissue biopsies are the golden standard in the pathological diagnosis, staging and treatment decision-making of malignant tumors, including primary lung cancer. However, tissue biopsies have several limitations [13, 18] (Fig. 2). First, tissue biopsies are an invasive procedure and may not be always applicable to patients with advanced cancer. Second, even when tissue biopsies can be performed, they may represent only a snapshot of tumor at the time of biopsies. Finally, the tumor characteristics may be heterogeneous not only among tumor sites in the same patient ("inter-tumor heterogeneity") but also among different areas of the same tumor ("intra-tumoral heterogeneity"). Tumor heterogeneity may be enhanced after treatment, which may make it difficult to overcome resistance

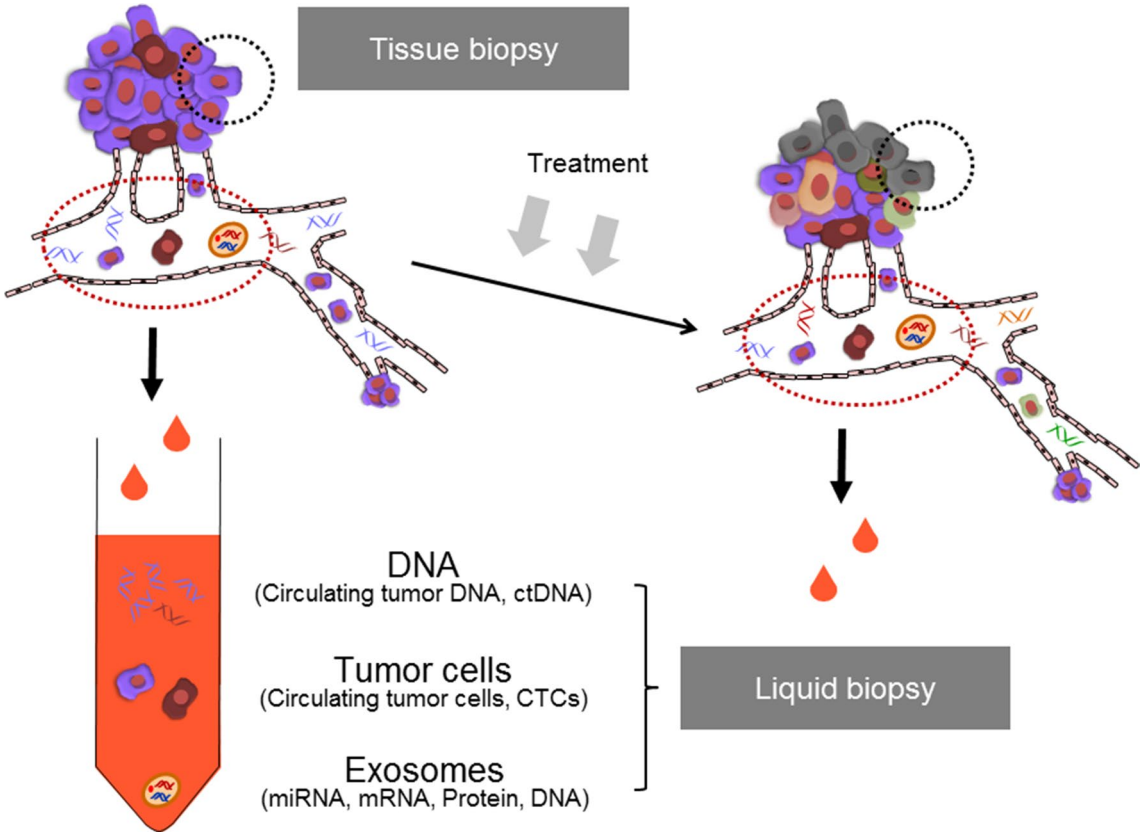

Fig. 2 Tissue biopsies versus liquid biopsies. Pathological and molecular information at the biopsy site of a tumor can be obtained with tissue biopsies. Peripheral blood may contain tumor cells as well as tumor-derived DNA and extracellular vesicles (exosomes) that come from all tumors in the whole body, so the heterogeneous tumor characteristics can be non-invasively monitored with liquid biopsies. *DNA* deoxyribonucleic acid, *RNA* ribonucleic acid

Table 1 Clinical applications of blood-based liquid biopsies Modified from Table 1 in [16]

Approach	Applications
Diagnostic	Early detection
	Monitoring of minimal residual disease (MRD)
Predictive	The assessment of molecular heterogeneity of overall disease
	The identification of genetic determinations for targeted therapy
	The evaluation of early treatment response
	The assessment of the evolution of resistance in real time
Prognostic	The identification of a high risk of recurrence
	The correlation with changes in the tumor burden

to treatment. Tissue biopsies only provide pathological and molecular information of the biopsied parts of tumor, which are not always representative of the global tumor characteristics.

The peripheral blood of cancer patients may contain tumor cells shed from tumor tissues (circulating tumor cells, CTCs) and DNA fragments derived from tumor cells (circulating tumor deoxyribonucleic acid, ctDNA), both of which can be used for tumor characterization. Exosomes, extracellular vesicles released from tumor cells, in the peripheral blood are also useful for liquid biopsies, as exosomes may contain micro-ribonucleic acid (miRNA), messenger RNA (mRNA), protein and DNA derived from tumor cells (Table 2) [9, 16, 18–20]. In contrast to tumor tissues, peripheral blood can be easily and repeatedly sampled from patients, which may allow serial sampling to monitor tumor characteristics during treatment ("real-time biopsies"). More importantly, liquid biopsies may overcome tumor heterogeneity, as the peripheral blood may

Table 2 A comparison of the methods for blood-based liquid biopsies

	Circulating tumor DNA (ctDNA)	Circulating tumor cells (CTCs)	Exosomes
Applications			
DNA analysis	Yes	Yes	Yes
RNA analysis	No	Yes	Yes
Protein analysis	No	Yes	Yes
Cell analysis	No	Yes	No
Advantages	High sensitivity in the detection of DNA alterations (somatic mutations, insertions and deletions, copy number alterations, gene fusions)	Direct quantitative analyses of tumor cells with high specificity Morphological, molecular and further biological analyses of isolated tumor cells	Analyses of RNA (miRNA, mRNA, long-coding RNA, RNA-specific variant, RNA expression) Utility for drug delivery as vectors
Limitations	Difficult to distinguish ctDNA from circulating DNA derived from normal cells No functional analysis	Relatively low sensitivity and/or reproducibility in isolation of tumor cells (technical challenge)	Difficult to distinguish tumor-derived exosomes from exosomes derived from normal cells Difficulty in extraction

DNA deoxyribonucleic acid, *RNA* ribonucleic acid, *miRNA* micro-RNA, *mRNA* messenger RNA

contain tumor cells and/or tumor-derived information from all of the tumors in a patient (Fig. 2).

Cell-based liquid biopsies versus cell-free liquid biopsies

Despite their advantages over tissue biopsies, liquid biopsies remain only an alternative to tissue biopsies mainly due to their low sensitivity and/or reproducibility. Cell-based liquid biopsies to capture CTCs have potential advantages over cell-free liquid biopsies to analyze ctDNA or exosomes, as the direct morphological and quantitative evaluation of tumor cells circulating in the peripheral blood can be performed. In addition, captured CTCs are subject to further molecular characterization, including protein analyses as well as DNA and RNA analyses (Table 2). For example, programmed death-ligand 1 (PD-L1) plays central roles in evasion from immune attack, and the status of its expression on tumor cells is an important predictor of the efficacy of agents targeting PD-1 and PD-L1 [8–10]. PD-L1 expression may be characterized by dynamic change in addition to intratumoral and inter-tumoral heterogeneity, and the tumoral PD-L1 status may be monitored with immune-histochemical staining of captured CTCs.

The CellSearch system is the only system approved for capturing CTCs [only by the Food and Drug Administration (FDA)], and it is used for monitoring the peripheral blood of patients with metastatic colorectal, breast and prostate cancer [21, 22]. However, while the CellSearch system may be useful for predicting the tumor progression and prognosis as well as the therapeutic efficacy in lung cancer [23, 24], its sensitivity for detecting CTCs seems to be insufficient for clinical use. In fact, a prospective study showed that no CTCs were detected in the peripheral blood in 9 (29.0%) of 31 patients with clinically detectable distant metastasis [23]. The development of a more-sensitive system for detecting CTCs with sufficient reproducibility is essential for the widespread introduction of cell-based liquid biopsies in clinical practice.

Polymerase chain reaction (PCR) is a useful tool for amplifying any DNA region of interest. Rare ctDNA fragments contaminating a vast amount of DNA derived from non-cancerous blood cells in the peripheral blood can be amplified with PCR, and thus be used to perform sensitive analyses of various gene alterations, including somatic mutations, insertions and deletions, copy number alterations and gene fusions. Recent technical advances, such as a variety of real-time PCR-based assays, have allowed for blood-based liquid biopsies analyzing ctDNA ("cell-free liquid biopsies") to be introduced into routine clinical practice. More recently, digital PCR platforms, including the droplet digital PCR (ddPCR) and beads, emulsion, amplification, and magnetics (BEAMing), have enabled the more-sensitive detection of specific gene alterations. In addition, newer platforms based on next-generation sequencing (NGS) have allowed for simultaneous multiplexed analyses of a wide range of gene alterations [13, 18].

Cell-free liquid biopsies

ctDNA and its analysis

Tumor cells may release DNA fragments into the circulation through apoptosis, necrosis and active secretion. Circulating DNA released from tumor cells (ctDNA) is

highly fragmented. The length of fragmented ctDNA is often below 200 base pairs, which suggests that ctDNA is released into the circulation mainly through apoptosis [16, 25–27]. It has been shown that ctDNA is rapidly cleared from the circulation, with estimated half-life times of 16 min and 2.5 h, mainly through nuclease digestion and renal excretion into the urine [16, 25]. Accordingly, the tumor burden and molecular characteristics can be monitored in real-time as a snapshot by analyzing ctDNA in the peripheral blood serially sampled from a patient.

Given that a large amount of DNA fragments released from non-cancerous cells, mainly from normal blood cells, also circulate in the peripheral blood, DNA fragments derived from tumor cells (ctDNA) usually comprise only a small fraction (< 1%) of the total DNA fragments circulating in the peripheral blood (cell-free DNA, cfDNA) [25, 26]. Accordingly, the reliable detection of gene alterations in ctDNA is a technical challenge. Appropriate pre-analytical conditions, including sample preparation and storage, are important (Fig. 3), and a high sensitivity of detection in assays is mandatory (Fig. 4).

Pre-analytical conditions (Fig. 3)

Pre-analytical conditions potentially affect the sensitivity, specificity, quantitation and reproducibility in cell-free liquid biopsies [18, 28–30]. For example, any loss or degradation of mutated DNA fragments during the pre-analytical processes can cause "false-negative" results, and patients may not be treated with optimum agents (for example, EGFR-TKIs for *EGFR*-mutated patients). Both plasma and serum contain ctDNA fragments and can be used for ctDNA analyses. However, during the preparation of serum, normal blood cells lyse and release germ-line DNA fragments. As a result, the serum may contain a higher total amount of DNA fragments, with tumor-derived ctDNA fragments diluted in the plasma. Accordingly, plasma is preferred over serum for ctDNA analyses, as rare gene alterations in ctDNA may be hidden in the background of a vast majority of wild-type DNA in the serum [18, 27, 28]. Indeed, two comparative studies for the detection of activating *EGFR* mutations indicated a higher sensitivity when plasma was used for ctDNA analyses (sensitivity, 94.7% for plasma versus 72.2% for serum [31]; 48.2 versus 39.6% [32]).

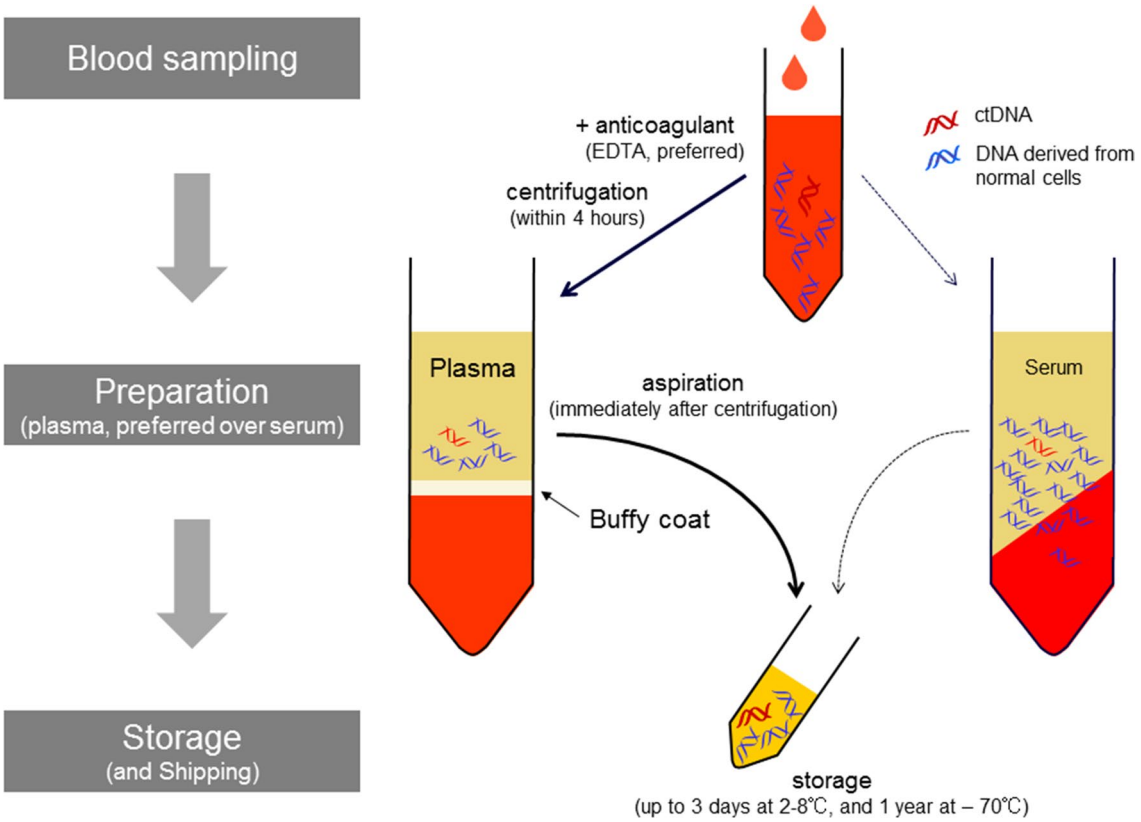

Fig. 3 Pre-analytical conditions for analyzing ctDNA. For ctDNA analyses, plasma is preferred over serum, as the larger amount of contaminated wild-type DNA derived from normal cells in the serum may hide the presence of rare mutated DNA. As an anticoagulant for plasma preparation, EDTA is recommended, as heparin may inhibit downstream PCR amplification. *DNA* deoxyribonucleic acid, *ctDNA* circulating tumor deoxyribonucleic acid, *EDTA* ethylenediaminetetraacetic acid, *PCR* polymerase chain reaction

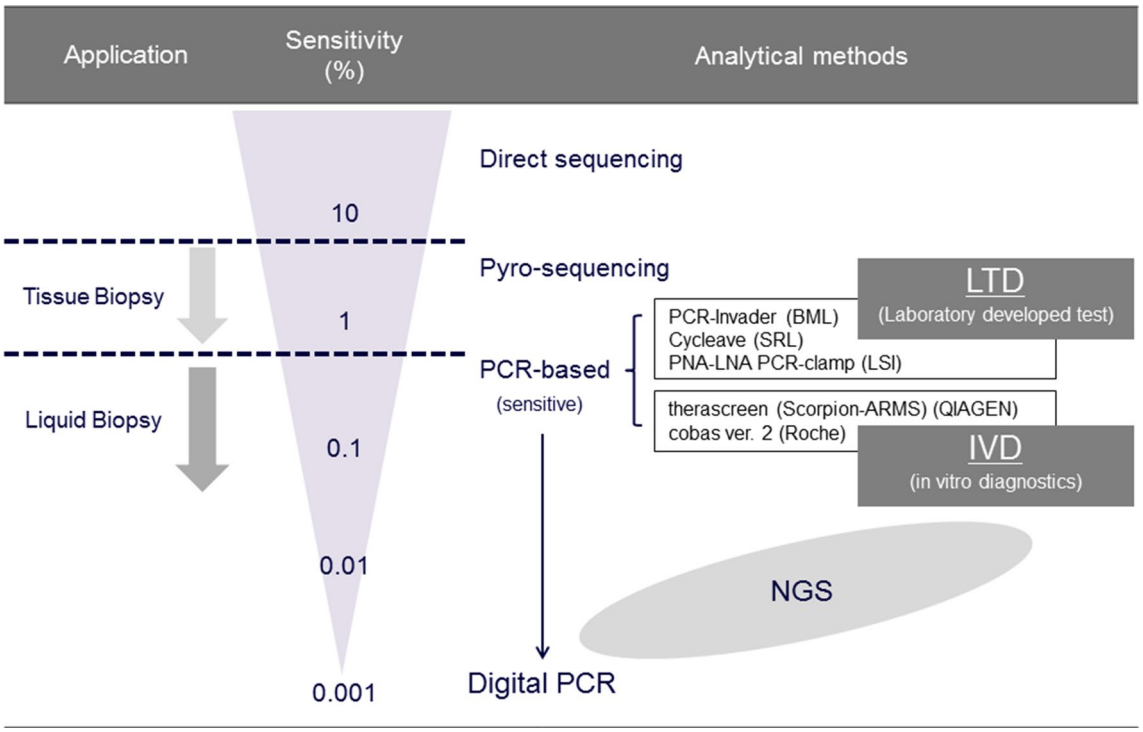

Fig. 4 Analytical methods and sensitivity in the detection of gene alterations. Standard sequencing assays, such as direct sequencing and pyro-sequencing, do not have high sensitivity (< 1%), which is mandatory in the detection of rare gene alterations derived from tumors in the plasma. Sensitive real-time PCR-based assays, such as cobas (version 2) and therascreen, are currently indicated for plasma genotyping. Digital PCR methods have a high sensitivity (≤ 0.001%), and NGS-based assays have enabled the simultaneous detection of multiple gene alterations. *PCR* polymerase chain reaction, *PNA–LNA* peptide nucleic acid–locked nucleic acid, *NGS* next-generation sequencing

To prepare plasma, sampled blood shall be immediately collected in a tube containing an anticoagulant. As an anticoagulant, ethylenediaminetetraacetic acid (EDTA) is preferred, as heparin may interfere with downstream PCR amplification. It is important to perform centrifugation for plasma preparation within 4 h of sampling to prevent the contamination of germ-line DNA fragments caused by the degradation of normal blood cells during longer-term storage. For the same reason, the plasma layer should be harvested immediately after centrifugation. Fresh plasma can be stored for up to 3 days at 2–8 °C but should be stored at − 80 °C for long-term storage [28, 29].

Methods of analyzing ctDNA (Fig. 4)

The sensitivity achieved with standard sequencing approaches, such as direct DNA sequencing and pyro-sequencing, may not be sufficient for liquid biopsies as well as tissue biopsies. A variety of PCR-based assays with increased sensitivity for the detection of gene alterations, such as activating *EGFR* mutations, have been developed and are currently available for tissue genotyping.

Among them, an allele-specific real-time PCR-based assay (combas® EGFR Mutation Test version 2; Roche Molecular Systems, Pleasanton, CA, USA) has been approved as an in vivo diagnostic (IVD) test to detect activating and resistant *EGFR* mutations in the plasma. Another allele-specific real-time PCR-based assay (therascreen® EGFR RGQ PCR kit; QIAGEN, Valencia, CA, USA) using Scorpion primers and an amplification-refractory mutation system (ARMS) has also been approved as a plasma IVD test to select patients with activating EGFR mutations for gefitinib administration (only in the European Union). These sensitive PCR-based assays may be useful for identifying the presence of activating *EGFR* mutations in the plasma with high specificity (93.5% in a meta-analysis) [33]. However, low sensitivity (67.4%) may be a critical issue when deciding on systemic treatment [33], as *EGFR*-mutated patients with "false-negative" results (*EGFR*-mutation, not detected in the plasma) may not be treated with the optimum agent (EGFR-TKI). A large-scale study in a real-world setting (ASSESS study) also showed a high specificity (97%) in detecting activating *EGFR* mutations in plasma sampled from advanced NSCLC patients [34]. However, the sensitivity was still relatively low (46%) [34], which may suggest

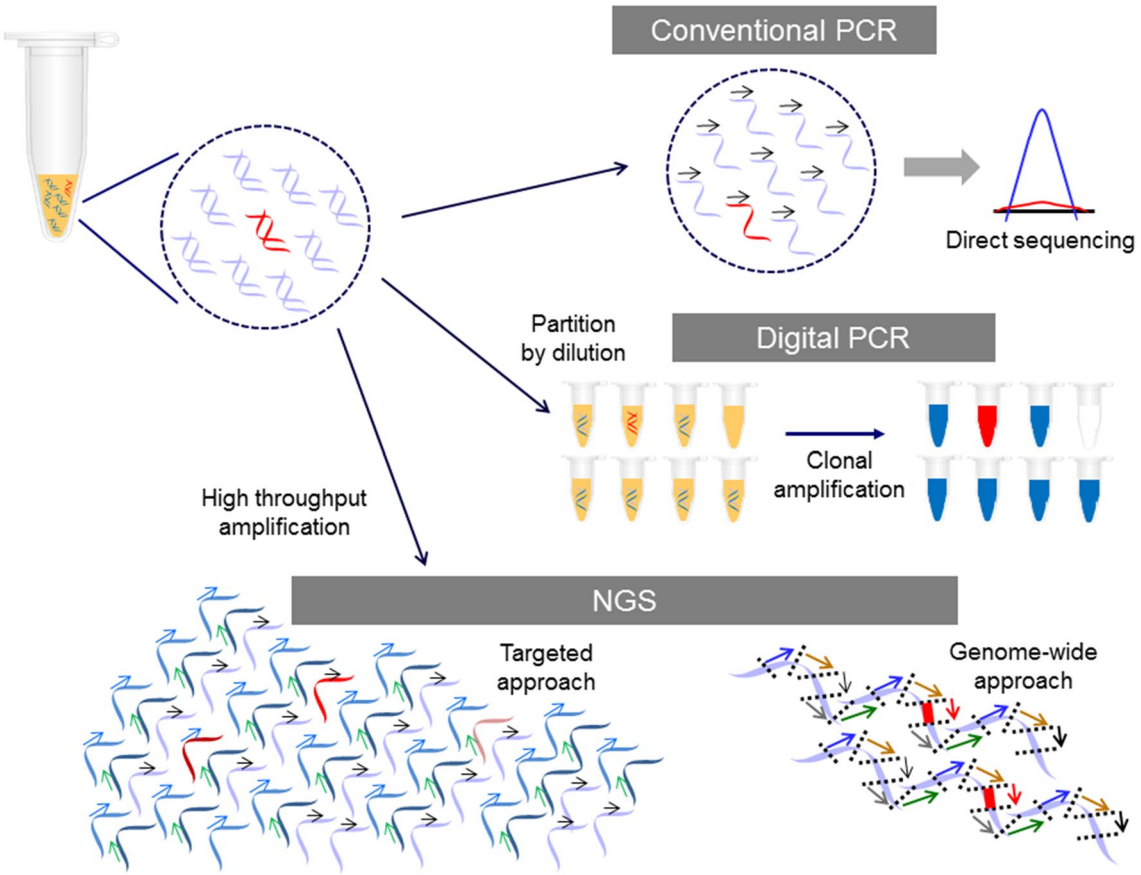

Fig. 5 Conventional PCR versus digital PCR and NGS. Clonal DNA amplification with digital PCR enables the absolute and accurate quantification, which provides a high sensitivity in the detection of gene alterations of interest. High-throughput sequencing with NGS enables simultaneous multiplexed analyses of a wide range of gene alterations. Non-targeted NGS-based platforms are applicable for genome-wide analyses. *DNA* deoxyribonucleic acid, *PCR* polymerase chain reaction. *NGS* next-generation sequencing

that the pre-analytical conditions are not optimum in a real-world setting.

Digital PCR is a new technology for clonally amplifying DNA that has enabled the absolute and precise quantitation of a target gene region (Fig. 5). In digital PCR, the sample is divided into a large-number of separate partitions, such as oil droplets in ddPCR [35, 36] and emulsions in BEAMing [37, 38], for which each partition contains either a single template DNA molecule or none. Clonal amplification with digital PCR has allowed for the detection of single-mutated DNA fragment contaminated in a large amount of wild-type DNA fragments [35–38]. This high sensitivity, reaching detection limits below 0.001%, may be sufficient for the detection of rare alterations in ctDNA.

NGS, or high-throughput sequencing, involves the massive and parallel sequencing of millions of DNA fragments, and several NGS-based platforms have been developed [39, 40] (Fig. 4). Deep-sequencing platforms for analyzing targeted genes have enabled the highly sensitive detection of known alterations in genes. Non-targeted genome-wide platforms have enabled the identification of a variety of tumor-specific gene alterations without knowledge of the alterations before the analysis. The development of NGS-based assays has provided new insights into ctDNA analyses, and the most important advantage is the ability to simultaneously detect multiple gene alterations, which may be very useful for monitoring tumor heterogeneity [13, 18, 39, 41–43]. For example, a targeted deep-sequencing platform to analyze common gene alterations found in cancer (Ion AmpliSeq™Cancer Hotspot Panel, Waltham, MA, USA) can survey a variety of alterations in 207 hot-spot regions across 50 genes, including *EGFR, ALK, BRAF, RET, PTEN* and *TP53*.

Plasma genotyping in NSCLC

A number of retrospective and prospective studies on plasma genotyping in NSCLC have been reported [14, 33, 44–47]. From the perspective of clinical practice, we focused on the

Table 3 The detection of activating *EGFR* mutations in plasma of NSCLC patients at baseline (before treatment with EGFR-TKI)

Study	Mutations	Sensitivity	Specificity	Concordance
DHPLC (denaturing high-performance liquid chromatography)				
Bai [48]	*EGFR* Del 19/L858R	81.8% (63/77)	89.5% (137/153)	87.0% (200/230)
Mass spectrometry genotyping assay (sequenom)				
Brevet [49]	*EGFR* Del 19/L858R	44.4% (8/18)	84.6% (11/13)	61.3% (19/31)
	EGFR Del 19	35.7% (5/14)	88.2% (15/17)	64.5% (20/31)
	EGFR L858R	75.0% (3/4)	100% (27/27)	96.8% (30/31)
High-resolution melting (HRM) assay				
Hu [50]	*EGFR* Del 19/L858R/other activating mutations	91.7% (22/24)	NA	NA
PNA (peptide nucleic acid)-mediated PCR (polymerase chain reaction) clamping				
Kim [51]	*EGFR* Del 19/L858R	17.1% (6/35)	100% (5/5)	27.5% (11/40)
Karachaliou and EURTAC study [52]	*EGFR* Del 19/L858R	78.4% (76/97)	100%	NA
	EGFR Del 19	83.9% (47/56)	100%	NA
	EGFR L858R	70.7% (29/41)	100%	NA
Therascreen (SCORPION-ARMS)				
Kimura [53]	*EGFR* Del 19/L858R	85.7% (6/7)	94.3% (33/35)	92.9% (39/42)
Goto and IPASS study [54]	*EGFR* Del 19/L858R	43.1% (22/51)	100% (35/35)	66.3% (57/86)
Douillard and IFUM study [55]	*EGFR* Del 19/L858R	65.7% (69/105)	99.8% (546/547)	94.3 (615/652)
	EGFR Del 19	67.6% (48/71)	100% (581/581)	96.5% (629/652)
	EGFR L858R	61.8% (21/34)	99.8% (617/618)	97.9% (638/652)
Wu and LUX-Lung3 study [56]	*EGFR* Del 19	33.3% (49/147)	97.0% (131/135)	63.8% (180/282)
	EGFR L858R	21.8% (26/119)	98.8% (160/162)	66.2% (186/281)
Wu and LUX-Lung6 study [56]	*EGFR* Del 19	64.0% (112/175)	97.4% (149/153)	79.9% (262/328)
	EGFR L858R	55.1% (70/127)	99.5% (200/201)	82.3% (270/328)
Cobas				
Weber [57]	*EGFR* Del 19/L858R/Ins 20	60.7% (17/28)	96.4% (162/168)	91.3% (179/196)
	EGFR Del 19/L858R	62.9% (17/27)	96.4% (163/169)	91.8% (180/196)
Mok and FASTACT-2 study [58]	*EGFR* Del 19/L858R/other activating mutations	75.0% (72/96)	96.5% (137/142)	87.8% (209/238)
	EGFR Del 19	82.5% (47/57)	98.3% (178/181)	94.5% (225/238)
	EGFR L858R	62.2% (23/37)	99.0% (199/201)	93.3% (222/238)
Reckamp and TIGER-X study [59] **patients who progressed on EGFR-TKI, included	*EGFR* Del 19/L858R/other activating mutations	73% (55/75)	100% (24/24)	80% (79/99)
	EGFR Del 19	71% (29/41)	100% (54/54)	87% (83/95)
	EGFR L858R	78% (21/27)	100% (68/68)	94% (89/95)
Cobas version 2				
FDA and ENSURE study [60]	*EGFR* Del 19/L858R	76.7% (161/210)	98.2% (217/221)	87.7% (378/431)
	EGFR Del 19	80.8% (97/120)	98.7% (307/311)	93.7% (404/431)
	EGFR L858R	67.8% (61/90)	99.1% (338/341)	92.6% (399/431)
Microfluidics digital PCR				
Yung [61]	*EGFR* Del 19/L858R *tumor mutation status determined with sequencing as reference	91.7% (11/12)	100% (17/17)	96.6% (28/29)
	EGFR Del 19/L858R *tumor mutation status determined with digital PCR as reference	78.9% (15/19)	100% (16/16)	88.6% (31/35)
Digital PCR (droplet digital PCR, ddPCR)				
Lee [62] KLCC-12-02 study	*EGFR* Del 19	76.5% (26/34)	100% (24/24)	86.2% (50/58)
	EGFR L858R	70.8% (17/24)	100% (34/34)	87.9% (51/58)

Table 3 (continued)

Study	Mutations	Sensitivity	Specificity	Concordance
Sacher [63]	*EGFR* Del 19/L858R	77.8% (21/27)	100% (203/203)	97.4% (224/230)
	EGFR Del 19	85.7% (12/14)	100% (101/101)	98.3% (113/115)
	EGFR L858R	69.2% (9/13)	100% (102/102)	96.5% (111/115)
NGS (IonTorrent-based deep-sequencing)				
Couraud and BioCAST/IFCT-1002 study [64]	*EGFR/KRAS/BRAF/ERBBB2/PI3KCA*	57.7% (26/45)	87.0% (20/23)	67.6% (46/68)
	EGFR Del 19	55.0% (11/20)	94.9% (37/39)	81.4% (48/59)
	EGFR L858R	71.4% (5/7)	100% (54/54)	96.7% (59/61)
NGS (deep-sequencing)				
Uchida [65]	*EGFR* Del 19/L858R	54.4% (56/103)	NA	NA
	EGFR Del 19	50.9% (27/53)	98.0%	NA
	EGFR L858R	51.9% (27/52)	94.1%	NA
NGS ("bias-corrected" targeted sequencing)				
Paweletz [66]	*EGFR/KRAS/BRAF/HER2/PIK3CA/ALK ALK/ROS1/RET/MET/MEK1/TP53*	77.4% (48/62)	100%	NA
	EGFR Del 19/L858R	66.7% (6/9)	100% (24/24)	90.9% (30/33)
NGS (Guardant360)				
Thompson [67] **patients who progressed on EGFR-TKI, included	*EGFR* Del 19/L858R/other activating mutations	79.2% (19/24)	100% (26/26)	90.0% (45/50)

EGFR epidermal growth factor receptor, *TKI* tyrosine kinase inhibitor, *NSCLC* non-small cell lung cancer, *NA* not assessed, *NGS* next-generation sequencing

use of ctDNA for the detection of activating and resistant *EGFR* mutations and reviewed only studies with retrospective or prospective clinical validation [47–72].

The detection of activating EGFR mutations in plasma

Before the initiation of treatment with EGFR-TKIs, plasma genotyping with current real-time PCR-based assays provides modest sensitivity and excellent specificity (over 95%) in the detection of common activating *EGFR* mutations, such as Del 19 and L858R (Table 3) [48–67]. For example, in a prospective phase IV study of first-line gefitinib treatment for *EGFR*-mutated advanced NSCLC patients (IFUM study), the sensitivity and specificity with the therascreen assay were 65.7 and 99.8%, respectively [55]. In a retrospective analysis of a phase 3 randomized controlled trial comparing erlotinib with chemotherapy (cisplatin plus gemcitabine) for *EGFR*-mutated advanced NSCLC (ENSURE) [73], the sensitivity and specificity with the cobas (version 2) assay were 76.7 and 98.2%, respectively [60].

Digital PCR assays, as expected, have achieved increased sensitivity for the detection of activating *EGF*R mutations without increasing the frequency of false-positive results (Table 3). For example, in a prospective validation study, ddPCR demonstrated 77.8% sensitivity and 100% specificity [63]. NGS-based assays may be useful for the simultaneous detection of a wide variety of gene alterations. At present, several NGS-based platforms, such as Guardant360 (Guardant Health, Redwood City, CA, USA) [43] and FoundationACT (Foundation Medicine, Cambridge, MA, USA) [74], are commercially available. These NGS-based assays have enabled the simultaneous detection of multiple gene alterations, including activating *EGFR* mutations, with high sensitivity comparable to that of digital PCR-based assays (Table 3).

Activating *EGFR* mutations may be detected in the plasma even after the initiation of treatment with EGFR-TKIs, usually at tumor progression (Table 4) [59, 62–64, 68–72]. For example, a prospective validation study of plasma genotyping with ddPCR showed similar sensitivities in the detection of activating *EGFR* mutations before the initiation of EGFR-TKI treatment as well as at tumor progression (77.8 and 79.6%, respectively). Of note, no "false-positive" results were documented in either sample (specificity, 100%) [73].

Detection of a resistant EGFR mutation (T790M) in plasma

Although EGFR-TKIs are effective for treating NSCLC with activating EGFR mutations, most patients treated with EGFR-TKIs show tumor progression around 1 year after the initiation of the treatment [6, 8]. A second *EGFR* mutation

Table 4 The detection of activating *EGFR* mutations in plasma of NSCLC patients after treatment with EGFR-TKI

Study	Mutations	Sensitivity	Specificity	Concordance
Cobas version 2				
Jenkins and AURA extension study (phase II) + AURA2 study [68]	*EGFR* Del 19	85%	98%	90%
	EGFR L858R	76%	98%	91%
Digital PCR (droplet digital PCR, ddPCR)				
Sacher [63]	*EGFR* Del 19/L858R	79.6% (43/54)	100% (64/64)	90.7% (107/118)
	EGFR Del 19	80.6% (29/36)	100% (23/23)	88.1% (52/59)
	EGFR L858R	77.8% (14/18)	100% (41/41)	93.2% (55/59)
Takahama and WJOG8014LTR study [69]	*EGFR* Del 19/L858R	75.8% (25/33)	87.5% (7/8)	78.0% (32/41)
Lee and KLCC-12-02 study [62]	*EGFR* Del 19	75.0% (21/28)	100% (21/21)	85.7% (42/49)
	EGFR L858R	52.4% (11/21)	100% (28/28)	79.6% (39/49)
Digital PCR (beads, emulsification, amplification, and magnetics digital PCR, BEAMing)				
Mok and IMPRESS study [70]	*EGFR* Del 19 *tumor mutation status at baseline (before TKI treatment) as reference	73.8% (124/168)	96.7% (89/92)	81.9% (213/260)
	EGFR L858R *tumor mutation status at baseline (before TKI treatment) as reference	81.6% (62/76)	95.3% (161/169)	91.0% (223/245)
Karlovich and TIGER-X study [71]	*EGFR* Del 19/L858R/other activating mutations	81.7% (49/60)	66.7% (2/3)	81.0% (51/63)
Oxnard and AURA study (phase I) [72]	*EGFR* Del 19	82.3% (112/136)	97.5% (78/80)	88.0% (190/216)
	EGFR L858R	70.3% (63/73)	96.5% (138/143)	93.1% (201/216)
NGS ("bias-corrected" targeted sequencing)				
Paweletz [66]	*EGFR* Del 19/L858R	100% (12/12)	100% (2/2)	100% (14/14)
NGS (short footprint mutation enrichment next-generation sequencing)				
Reckamp and TIGER-X study [59]	*EGFR* Del 19	87.2% (34/39)	95.9% (47/49)	92.0% (81/88)
	EGFR L858R	100% (17/17)	100% (48/48)	100% (65/65)

EGFR epidermal growth factor receptor, *TKI* tyrosine kinase inhibitor, *NSCLC* non-small cell lung cancer, *PCR* polymerase chain reaction, *NGS* next-generation sequencing

in exon 20 (T790M) is responsible for the acquired resistance in approximately 50–60% of patients who progress while on treatment with first- or second-generation EGFR-TKIs [75–77]. To overcome this acquired resistance caused by T790M, several third-generation EGFR-TKIs have been developed. Among them, osimertinib is the only approved agent, based on the accumulation of data indicating its robust clinical activity against *EGFR*-mutated NSCLC with T790M in several clinical trials (AURA extension and AURA2) [78, 79]. A phase 3 randomized control trial (AURA3) confirmed the efficacy of osimertinib, showing its superior survival benefit over platinum-based chemotherapy for patients with acquired resistance to EGFR-TKIs caused by T790M [80]. Accordingly, tumor biopsies should be performed to examine the resistance mechanism, and liquid biopsies should therefore be considered as an alternative approach to detect T790M in the plasma (Table 5) [59, 63, 66, 68, 69, 71, 72].

In a retrospective analysis of plasma ctDNA for detection of T790M in patients enrolled on two phase 2 studies of osimertinib (AURA extension and AURA2), the cobas (version 2) test showed comparable sensitivity (61.3%) but lower specificity (78.6%) than in the detection of activating *EGFR* mutations (98%) [68]. A prospective validation study with ddPCR showed a higher sensitivity (77.1%) in the detection of T790M, but the specificity was still lower than that for the detection of activating EGFR mutations (63.2%) [70]. High incidences of "false-positive" results (T790M-positive in the plasma but T790M-negative in the tumor) may be due to a heterogeneous resistance mechanism. Plasma genotyping, especially with sensitive detection assays, such as ddPCR, has an advantage when evaluating tumor heterogeneity, but the clinical relevance of detected gene alterations should be carefully investigated. Indeed, the response rate of osimertinib in "false-positive" patients was only 28%, whereas the response rate for T790M-positive by tissue biopsies was over 60%, regardless of the positivity of plasma genotyping [72].

Table 5 The detection of resistant *EGFR* mutation (T790M) in the plasma of NSCLC patients

Author and study	Sensitivity	Specificity	Concordance
(1) T790M detection at baseline (before EGFR-TKI treatment)			
Therascreen (SCORPION-ARMS)			
Wu and LUX-Lung3 study [56]	30.0% (3/10)	99.6% (264/265)	97.1% (267/275)
Wu and LUX-Lung6 study [56]	0% (0/1)	99.7% (326/327)	99.4% (326/328)
NGS (Guardant360)			
Thompson [67] **patients who progressed on EGFR-TKI included	50.0% (2/4)	87.0% (40/46)	84.0% (42/50)
(2) T790M detection after EGFR-TKI treatment			
Cobas version 2			
Jenkins and AURA extension study (phase II) + AURA2 study [68]	61.3% (254/414)	78.6% (99/126)	65.3% (353/540)
Digital PCR (droplet digital PCR, ddPCR)			
Sacher [63]	77.1% (27/35)	63.2% (12/19)	68.4% (39/54)
Takahama and OG8014LTR study [69]	64.5% (20/31)	70.0% (7/10)	65.9% (27/41)
Digital PCR (beads, emulsification, amplification, and magnetics digital PCR, BEAMing)			
Karlovich and TIGER-X study [71]	73.3% (33/45)	50.0% (9/18)	66.7% (42/63)
Oxnard and AURA study (phase I) [72]	70.3% (111/158)	69.0% (40/58)	69.9% (151/216)
NGS ("bias-corrected" targeted sequencing)			
Paweletz [76]	81.8% (9/11)	100% (3/3)	85.7% (12/14)
NGS ("short footprint mutation enrichment next-generation sequencing)			
Reckamp and TIGER-X study [59]	92.7% (38/41)	93.8% (60/64)	93.3% (98/105)

EGFR epidermal growth factor receptor, *TKI* tyrosine kinase inhibitor, *NSCLC* non-small cell lung cancer, *PCR* polymerase chain reaction, *NGS* next-generation sequencing

Conclusions

Liquid biopsies have several advantages over tissue biopsies, which are the golden standard for evaluating the molecular characteristics of tumors. Recent advances in molecular analyses have enabled liquid biopsies to be introduced into daily clinical practice. For example, the detection of activating and sensitive *EGFR* mutations in plasma has been approved, which may aid in selecting patients treated with EGFR-TKIs. However, liquid biopsies have several limitations, such as the fact that "false-negative" results can be caused by inappropriate pre-analytical conditions. In addition, it is difficult to detect EGFR-TKI resistance caused by pathological transformation with PCR-based liquid biopsies, although this can be confirmed with tissue biopsies. Physician should be aware of the limitations as well as the advantages of liquid biopsies and should provide the optimum treatment to individual patients by carefully evaluating the results of biopsies.

Compliance with ethical standards

Conflict of interest Kazue Yoneda has no conflicts of interest; Naoko Imanishi has no conflicts of interest; Yoshinobu Ichiki has no conflicts of interest; Fumihiro Tanaka received a research grant from Astra Zeneca, Chugai Pharmaceutical, Taiho Pharmaceutical and Ono Pharmaceutical and received honoraria from Astra Zeneca, Chugai Pharmaceutical and Taiho Pharmaceutical.

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