



Circulating tumor DNA measurement provides reliable mutation detection in mice with human lung cancer xenografts

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Abstract

Genotype-directed targeted therapy has become one of the standard treatment options for non-small cell lung cancer (NSCLC). There have been numerous limitations associated with mutation analysis of tissue samples. Consequently, mutational profile analysis of circulating cell-free DNA (cfDNA) by highly sensitive droplet digital PCR (ddPCR) assay has been developed. Possibly due to differences in cfDNA concentrations, previous studies have shown numerous discrepancies in mutation detection consistency between tissue and cfDNA. In order to rigorously analyze the amount of cfDNA needed, we constructed 72 athymic nude mice xenografted with NCI-H1975 (harboring a *EGFR* T790M mutation) or NCI-H460 (harboring a *KRAS* Q61H mutation) human NSCLC. We thoroughly investigated the relationship between plasma cfDNA using Q-PCR targeting human long interspersed nuclear element-1 (*LINE-1*) retrotransposon and the mouse *ACTB* gene, and the accuracy of mutation detection by ddPCR at different times post-graft. Our results show that the concentration and fragmentation of human (tumor) derived cfDNA (hctDNA) were positively correlated with tumor weight, but not with mouse-derived cfDNA (mcfDNA). Quantification of cfDNA by Q-PCR depends on the amplified target length. Mutation copies in plasma of per milliliter were positively linked to tumor weight, hctDNA level and hctDNA/mcfDNA ratio, respectively. Furthermore, tumor weight, hctDNA level and ratio of hctDNA/mcfDNA were significantly higher in cfDNA mutation-positive mice than in negative mice. Also, our data indicate that when plasma hctDNA level and hctDNA/mcfDNA ratio reach a certain level in xenografted mice, plasma cfDNA mutation can be detected. In summary, the present study suggests that determination of ctDNA levels may be essential for reliable mutation detection by analysis of cfDNA.

Introduction

Non-small cell lung cancer (NSCLC), the most common cause of cancer-associated mortality worldwide, accounts for about 80% of all lung cancer. Various therapeutic agents have been developed to target signaling pathways in

NSCLC, including the epidermal growth factor receptor (EGFR) pathway. [1] Gefitinib and erlotini (two kinds of *EGFR* tyrosine kinase inhibitors), and cetuximab (a chimeric human mouse anti-EGFR monoclonal antibody) have been widely applied in clinical practice to treat cancer. The occurrence of point mutations, deletion or rearrangement of *EGFR* or Kirsten rat sarcoma viral oncogene homolog (*KRAS*), the key member of EGFR signaling pathway, have been shown to dramatically influence the therapeutic efficiency of targeted drugs. [2–5] Therefore, assessment of gene mutation status is mandatory in patients prior to targeted therapy. [6] Cancer tissues, including fresh and formalin-fixed, paraffin-embedded, are routinely applied as samples for gene mutation detection. However, the appropriate tissues for gene mutation analysis are rarely obtained, especially in patients with locally advanced or distant metastasis. Because of the multicenter origin of tumors, local sampling cannot reflect the tumor heterogeneity, while the single sampling cannot reflect tumor progression or possible changes in gene mutation status post-treatment.

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Therefore, it is improper to monitor gene mutation using the original surgical samples.

Circulating cell-free DNA (cfDNA), which mainly originates from apoptosis, necrosis and secretion of cells, has received substantial attention in recent years due to its multiple advantages as material for gene mutation detection. In cancer patients, cfDNA is mainly derived from tumor cells, whereas only a small part originates from normal host cells. [7] Tumor-derived cfDNA, also known as circulating tumor DNA (ctDNA), accounts for different fractions of cfDNA owing to differences in tumor burden. Because of its ability to overcome the problem of tumor heterogeneity and the ease of obtaining it non-invasively and dynamically, ctDNA has been used for mutation detection of *EGFR*, *KRAS*, *BRAF*, *APC*, *TP53*, in many types of cancers. [8–13] Nonetheless, several studies have shown the discordance of gene mutation status between tumor and matched blood samples. [14–17] Rosell et al. [14] reported that *EGFR* mutation status concordance in plasma and tissue samples was only 59.1%, while Jiang et al. [15] found it to be 93.1% in serum and tissues from 55 patients with advanced NSCLC using enrichment sequencing technology. Furthermore, Bai et al. [16] detected the *EGFR* mutations in paired tissue and plasma samples from 230 patients by denaturing high performance liquid chromatography (HPLC) assays and demonstrated that the results from 200 patients were consistent, with a concordance rate of 87%. Among 30 inconsistent cases, 16 patients displayed plasma DNA mutations, while 14 cases showed tissue mutations.

The reasons for inconsistent mutation detection may be due to two factors: First, the various methods have different sensitivity levels. The droplet digital PCR (ddPCR) assay, which is among the most sensitive technologies for gene mutation detection, has sensitivity that can reach 0.01%, i.e., it can detect one mutant copy in as many as 10,000 wild-type copies. Currently, ddPCR analysis has been widely used in mutation detection. [18–20] Second, the detection accuracy varies between the materials. Gene mutation that is detected in cfDNA, but not in corresponding tissues, can be attributed to improper sampling, tumor heterogeneity or cancer cells acquiring novel mutation; however, if it is detected in tissues but not found in cfDNA then this might mean it is not truly negative. Low amounts or excessive degradation of cfDNA revealing false-negative results should be taken in consideration. Isobe et al. [21] have shown that the detection rate of *EGFR* mutation in ctDNA was related to the number of circulating tumor cells (CTC). In patients with $CTC \geq 2/7.5$ ml and $CTC < 2/7.5$ ml from whole blood, the detection rate was 100% (4/4) and 10% (2/20), respectively. This study suggested that the quantity of ctDNA may be an important prerequisite for “liquid biopsy” used for successful mutation

detection. However, to date, there are no studies that have demonstrated the relationship between ctDNA concentration and mutation detection accuracy. Therefore, a concentration at which ctDNA can guarantee the reliability for mutation detection in genes still remains unclear.

The present study investigates an experimental system for systematical exploration of the above reported characteristics. The concentration of plasma cfDNA was quantified by quantitative-PCR (Q-PCR) in athymic nude mice xenografted with human NSCLC cells NCI-H1975 (harboring *EGFR* T790M mutation) or NCI-H460 (harboring *KRAS* Q61H mutation), as well as *EGFR* and *KRAS* mutation determined by ddPCR assay. This study aims to clarify the relationship between ctDNA concentration and mutation detection accuracy, and discusses the feasibility of previewing ctDNA concentration for reducing false-negative results in practical work.

Materials and methods

Cell lines and reagents

Human NSCLC cell lines, including lung adenocarcinoma NCI-H1975 (ATCC no. CRL-5908TM) and large-cell lung cancer NCI-H460 (ATCC no. HTB-177TM), were cultured in RPMI-1640 Medium (Gibco, USA) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. NCI-H1975 cells exhibited the heterozygous *EGFR* c.2369 C>T nucleotide mutation (amino acid change p.T790M), whereas NCI-H460 cells harbor a *KRAS* c.183 A>T nucleotide mutation (amino acid change p.Q61H). The kits of droplet ddPCR mutation assay for human *EGFR* c.2369 C>T mutation (Assay ID dHsaCP2000019 and dHsaCP2000020) and human *KRAS* c.183 A>T mutation (Assay ID dHsaCP2000131 and dHsaCP2000132) were purchased from Bio-Rad.

Subcutaneous tumor models

The flank regions of BALB/c nude mice (4–6 weeks old, female, from Beijing HFK Bioscience Co., Ltd., Beijing, China) were subcutaneously injected with 2×10^6 H1975 or H460 lung cancer cells in 0.1 ml of serum-free DMEM. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and killed with CO₂ at different time points post-graft. Peripheral blood was collected and transferred into tubes with EDTA-K2 as anticoagulant. The samples were used for plasma preparation within 1 h following sampling. Subcutaneous tumors were dissected and weighed. All animal experiments were approved by the Institutional Animal Care and Use Committee of Shandong Cancer Hospital and Institute, China.

Table 1 Primer sequences for human *LINE1* and mouse *ACTB* gene

| Species | Gene | Primer name | Primer sequence (5' → 3') Forward/reverse | Amplicon size |
|---------|--------------|-------------|---|---------------|
| Human | <i>LINE1</i> | hLINE 81 | TCACTCAAAGCCGCTCAACTAC TCTGCCTTCATTTTCGTTATGTACC | 81 bp |
| | | hLINE 297 | ACACCTATTCCAAAATTGACCAC TTCCCTCTACACACTGCTTTGA | 297 bp |
| Mouse | <i>ACTB</i> | mACTB 120 | GATGCACAGTAGGTCTAAGTGGAG CACTCAGGGCAGGTGAAACT | 120 bp |
| | | mACTB 338 | CTTCTGCCGTTCTCCCATAG CACTCAGGGCAGGTGAAACT | 338 bp |

Plasma preparation for quantitative polymerase chain reaction (Q-PCR)

Mice plasma samples were prepared using a two-step centrifugation process. First, mice blood specimens were centrifuged at $1600 \times g$ at 4°C for 10 min. Second, supernatants were isolated in a new sterile 1.5 ml Eppendorf tubes and then additionally centrifuged at $16,000 \times g$ at 4°C for 10 min to remove the remaining cellular debris. Lastly, the supernatants were either immediately used for DNA extraction or cryopreserved at -80°C . There were no obvious differences in Q-PCR assays between freshly isolated or stored plasma.

DNA extraction

Circulating DNA was extracted from plasma using QIAamp Circulating Nucleic Acid kit (Qiagen, Cat. no 55114) following the manufacturer's instructions. DNA samples were stored at -20°C until use. The genomic DNA from human NSCLC cells and peripheral blood leukocytes (PBL) of naive nude mice were purified using the QIAamp DNA Mini-Kit (Qiagen, Cat. no 51304) according to manufacturer's protocol. DNA concentration was evaluated with a spectrophotometer and was kept at -20°C until use. No significant difference was displayed in Q-PCR assay between freshly purified DNA and stored DNA.

cfDNA quantification by Q-PCR

SYBR Green based Q-PCR was used to quantify cfDNA extracted from plasma samples. Q-PCR assay consisted of UltraSYBR Mixture (CW0957, CWBIO, China), 200 nM primers, and 3 μL of DNA extract. The primer sequences used in this study referred to the published literature [13, 22, 23] and were displayed in Table 1. Human-specific primers were used for detection of human Long Interspersed Nuclear Element-1 (*LINE-1*) retrotransposon, generating an amplicon of 81 and 297 base pairs (hLINE 81 and hLINE 297), respectively. Mice-specific primers were used for the

detection of *ACTB* generating an amplicon of 120 and 338 base pairs (mACTB 120 and mACTB 338), respectively. All samples were measured in duplicates. Negative control (H_2O) was included in every run. Q-PCR reaction was monitored on a LightCycler 480 machine (Roche Applied Science, Mannheim, Germany) under the following cycle conditions: 95°C for 10 min followed by 40 repeated cycles of 95°C for 15 s and 60°C for 1 min. Serial dilutions of genomic DNA from H1975, H460 cells and PBL of normal nude mice were used as calibrators for human and mouse cfDNA quantification, respectively. The concentration of cfDNA was expressed with ng/ml plasma. Human (tumor) and mouse-derived cfDNA were named as hctDNA and mcfDNA, respectively. DNA integrity index (DII), calculated as the ratio of larger/shorter fragment concentrations, was used to evaluate cfDNA fragmentation degree. DII of human and mouse-derived cfDNA were abbreviated to hDII and mDII, respectively.

Pyrosequencing of NSCLC cells

Gene mutation validation of genomic DNA from H1975 and H460 cells were performed using pyrosequencing assay by Changsha Bio-3G technology Co., Ltd (Floor 3, Building A5, Luvalley National Hi-Tech Industrial Development Zone, Lugu Road, Changsha, 410205, Hunan Province, China).

Mutation copies in plasma cfDNA detected by ddPCR

Mutation copies of plasma cfDNA were determined by ddPCR with a QX200™ Droplet Digital™ PCR system (Bio-Rad, Hercules, CA, USA). PCR reaction solution contains 10 μL 2 \times ddPCR supermix (Bio-Rad), 1 μL 20 \times mutation primer/probe mixture (Probe Fluorophore FAM, Bio-Rad assay ID dHsaCP2000019 for *EGFR* or dHsaCP2000131 for *KRAS*), 1 μL 20 \times wild-type primer/probe mixture (Probe Fluorophore HEX, Bio-Rad assay ID dHsaCP2000020 for *EGFR* or dHsaCP2000132 for *KRAS*)

Table 2 Characteristics of xenografted mice with H1975 or H460 NSCLC cells

| Characteristic | H1975 xenografts (<i>n</i> = 36) | H460 xenografts (<i>n</i> = 36) |
|------------------------------|-----------------------------------|----------------------------------|
| Tumor weight (g) | 0.65 ± 0.52 (0.06–2.26) | 0.59 ± 0.31 (0.12–1.17) |
| hctDNA concentration (ng/ml) | | |
| hLINE 81 | 6.10 ± 21.35(0.37–186.76) | 73.36 ± 134.10 (0.37–813.25) |
| hLINE 297 | 0.34 ± 0.48 (0.01–9.39) | 9.54 ± 23.41 (0.09–82.70) |
| hDII | 0.05 ± 0.05 (0.01–0.28) | 0.17 ± 0.13 (0.05–0.32) |
| mcfDNA concentration (ng/ml) | | |
| mACTB 120 | 76.70 ± 33.10 (6.44–147.44) | 76.23 ± 30.25 (22.15–149.97) |
| mACTB 338 | 28.14 ± 11.81 (4.73–46.66) | 29.42 ± 9.67(11.26–49.73) |
| mDII | 0.39 ± 0.11 (0.19–0.74) | 0.44 ± 0.14 (0.16–0.79) |
| hctDNA/mcfDNA | 0.08 ± 0.32 (0.01–1.95) | 1.39 ± 1.78 (0.01–8.99) |

and 8 µL of cfDNA sample. This mixture was placed into the droplet generator DG8 cartridge (Bio-Rad), 70 µL of droplet generation oil was added, and droplets were formed in the droplet generator (Bio-Rad). Then, the droplets were loaded in a semi-skirted 96-well PCR plate (Eppendorf, Leuven, Belgium) and placed in a C1000 thermal cycler (Bio-Rad). PCR program was as follows: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 55 °C for 1 min, 1 cycle of 98 °C for 10 min, and ending at 4 °C. After amplification, the plates were read on a Bio-Rad QX200 Droplet reader for fluorescent measurement of FAM and HEX probes with the Quanta Soft v1.7 software (Bio-Rad). *KRAS* 183 A > T, 182 A > T mutation plasmid (Changsha Bio-3G technology Co., Ltd, Changsha, China) and *EGFR* T790M cfDNA reference standards (Horizon Discovery Ltd., Building 8100, Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom) were used as reference standards for *KRAS* and *EGFR* mutation assessment, respectively. No template control (NTC) was used to exclude PCR contamination. Each sample was analyzed in duplicate. Mutant copies of cfDNA in plasma were presented as copy number in 1 ml plasma. Samples with more than three positive mutant droplets were considered positive.

Statistical analysis

SPSS 13.0 software was used to perform statistical analysis. Data with skewed and normal distribution were presented as median ± interquartile range (IQR) (range, minimum-maximum) and mean ± SD (range, minimum-maximum), respectively. Bivariate correlation assay was adopted for correlation analysis. Mann–Whitney *U* test, a nonparametric test of two independent samples, was applied to compare differences between two groups of skewed distribution data. *P* value < 0.05 was considered statistically significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

Establishment of a standard curve for Q-PCR

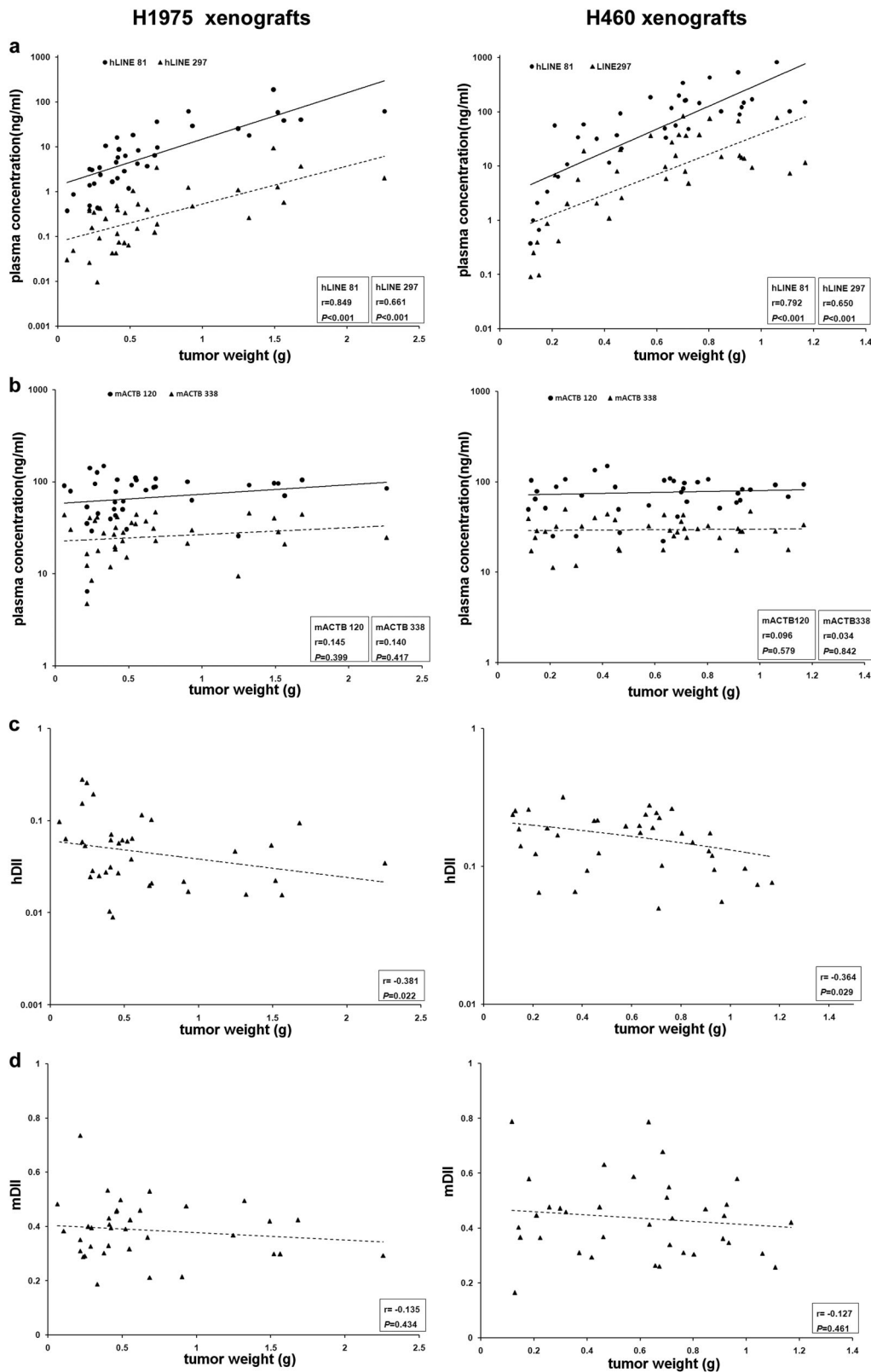
Quantification system of Q-PCR was assayed using serially diluted genomic DNA from H1975, H460 and PBL of normal nude mice and the following primer sets: hLINE 81, hLINE 297, mACTB 120 and mACTB 338. The amplification curve and standard curve of Q-PCR are shown in Supplementary Figure 1. Briefly, a good linear relationship was found between the log concentration of DNA amount (ng) and C_q values, and the R² for all PCR primer sets exceeded 0.99.

Specificity validation of Q-PCR primers

The primer specificity was assessed using plasma cfDNA from healthy control BALB/C nude mice and healthy individuals. Consistent with the previous publications, a very low amount (below 0.05 ng/ml) was observed with hLINE 81 and hLINE 297 primer in control mouse plasma. Similarly, no detectable amounts were observed with mACTB120 and mACTB338 in healthy human plasma. The above results confirmed that the primers used in this study have high Q-PCR amplification specificity (Supplementary Figure 2).

The concentration of hctDNA positively correlated with tumor weight

A total of 72 xenografted mice, including 36 mice injected with H1975 cells and 36 mice carrying H460 cells, were enrolled in the present study. The concentration of hctDNA and hDII displayed skewed distribution, while mcfDNA amount and mDII showed normal distribution. No matter hctDNA or mcfDNA, cfDNA concentrations using hLINE 81 and mACTB 120 primers were applied to



represent the actual amount of cfDNA. To further compare the proportion of tumor (human) derived and non-tumor (mouse) derived cfDNA, the ratio of concentration

using hLINE 81 and mACTB 120 primer sets (named as hctDNA/mcfDNA) were used. The ratio of hctDNA/mcfDNA displayed skewed distribution. The detailed

◀ **Fig. 1** Plasma cfDNA relative to tumor weight in xenografted mice carrying H1975 and H460 cells. The concentrations of plasma cfDNA were determined by Q-PCR analysis targeting human LINE1 and mouse ACTB sequences. DNA integrity index (DII) was calculated as the ratio of larger/shorter fragment concentrations. DII of human and mouse-derived cfDNA were abbreviated to hDII and mDII, respectively. Plasma ctDNA concentrations obtained with hLINE-1 (a), mACTB (b) primer sets, hDII (c) and mDII (d) were expressed versus tumor weight. Experiments were carried out in duplicate

features of xenografted mice with H1975 or H460 cells are summarized in Table 2.

Quantification of cfDNA by Q-PCR depends on the amplified target length. The concentrations of htDNA obtained with hLINE 81 primer were higher compared with hLINE 297 primer (17.9- and 7.7- fold higher for H1975 and H460 xenografts, respectively). In addition, htDNA concentrations determined by hLINE 81 and hLINE 297 primers in H460 xenografts were much higher compared to H1975 xenografts (12.0- and 28.1-fold higher for hLINE 81 and hLINE 297 primers, respectively). mcfDNA concentration, as well as mDII, showed similar levels between H1975 and H460 xenografts. Plasma htDNA/mcfDNA in H460 xenografts were obviously higher than that in H1975 xenografts (17.4-fold higher), indicating that the proportion of htDNA amount relative to mcfDNA level in H460 xenografts was much greater.

Scatter plots of plasma cfDNA in xenografted mice varying in relation to tumor weight are shown in Fig. 1. The data showed that htDNA amounts increased with tumor weight and were noticeably higher in mice with larger tumors in both H1975 or H460 xenografts (Fig. 1a). Nevertheless, mcfDNA concentrations were not influenced by tumor weight, indicating that mcfDNA level was relatively stable (Fig. 1b). Furthermore, the ratio of htDNA/mcfDNA showed positive correlations with tumor weight ($P < 0.001$). The Spearman's rho correlation coefficients between htDNA/mcfDNA value and tumor weight in H1975 and H460 xenografts were 0.782 and 0.662, respectively. As for hDII, it displayed negative correlation with tumor weight ($P < 0.05$), suggesting that htDNA fragmentation increases with tumor weight (Fig. 1c). mDII displayed no significant association with tumor burden (all $P > 0.1$) (Fig. 1d). To conclude, the above results demonstrated that htDNA may serve as a useful marker for monitoring tumor growth.

Mutation verification in H1975 and H460 cells by pyrophosphate sequencing

The mutation of *EGFR* T790M in H1975 cells and *KRAS* Q61H in H460 cells were determined by pyrophosphate sequencing technology. Nucleotide changes in H1975 and H460 cells were as follows: *EGFR* c.2369 C > T and *KRAS*

c.183 A > T. The results from pyrophosphate sequencing map indicated that mutation rates of *EGFR* in H1975 cells and *KRAS* in H460 cells were about 70 and 100%, respectively (Supplementary Figure 3).

Quality control of ddPCR assay for mutation analysis

Quality control of ddPCR assay for *EGFR* and *KRAS* mutation analysis was performed by Bio-Rad QX200™ Droplet Digital™ PCR system using positive control, negative control and no template control (NTC). In the cluster figure, fluorescent signals of mutation probe FAM and wild-type probe HEX were found on the upper left and low right regions, respectively. Consistent with the results of pyrophosphate sequencing, genomic mutation DNA statuses from H1975 and H460 cells were also confirmed by ddPCR assay. Representative figures of ddPCR detection are shown in Fig. 2a.

Mutated copies of plasma cfDNA positively correlated with tumor weight and htDNA concentration

Theoretically, because cancer cells were inoculated to each mouse, gene mutation could exist within the plasma of all xenografted mice. Regardless of H1975 or H460 xenografted mice, plasma ctDNA mutation copies all displayed skewness distribution. The median \pm IQR of mutation copies in 1 ml plasma in H1975 and H460 xenografted mice were 105.00 ± 426.00 (range, 0–8280) and 11160.00 ± 19264.50 (range, 0–148285.71), respectively. Representative ddPCR cluster diagrams from plasma ctDNA of xenografted mice are shown in Fig. 2b, c.

Scatter plots of mutational copies/ml plasma relative to tumor weight, htDNA, mcfDNA and htDNA/mcfDNA ratio are shown in Fig. 3. Spearman correlation analysis disclosed that mutant copies/ml plasma in xenografts were positively correlated with tumor weight, htDNA concentration and htDNA/mcfDNA ratio (all $P < 0.001$). Concentrations of mcfDNA were not correlated with mutant copies (all $P > 0.1$). According to the judgement standard of positive samples (more than three positive mutant droplets), the cases with positive mutations in H1975 and H460 xenografts were 16 and 32, respectively. Mann–Whitney *U* test displayed that either in H1975 or H460 xenografted mice, tumor weight, htDNA concentration and htDNA/mcfDNA ratio in mutation-positive groups were all significantly higher compared to mutation negative groups ($P < 0.01$). The detailed results are shown in Table 3. Subsequently, in order to observe whether tumor weight, htDNA concentration and htDNA/mcfDNA ratio influence the accuracy of mutation detection, the xenografted mice were divided into two groups. Briefly, the results shown in

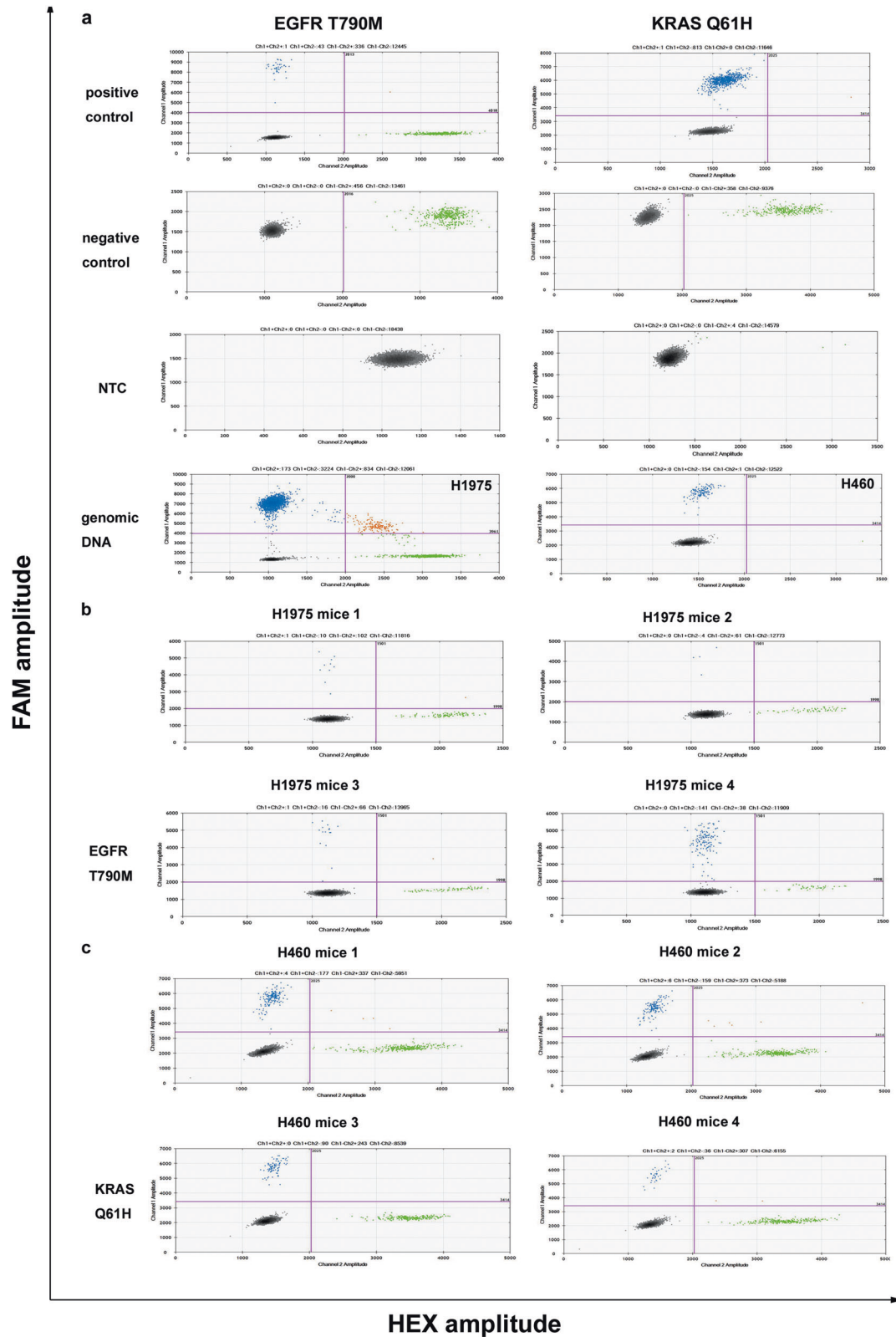


Fig. 2 Quality control of droplet digital PCR (ddPCR) assay and plasma cfDNA mutation detection. ddPCR was performed via Bio-Rad QX200™ Droplet Digital™ PCR system. Representative cluster figures for ddPCR mutation detection were displayed. The samples were as follows: **a** Positive control, negative control, no template control

(NTC), genomic DNA from H1975 and H460 cells. **b** *EGFR* T790M mutation detection of plasma cfDNA from H1975 xenografted mice. **(c)** *KRAS* Q61H mutation detection of plasma cfDNA from H460 xenografted mice

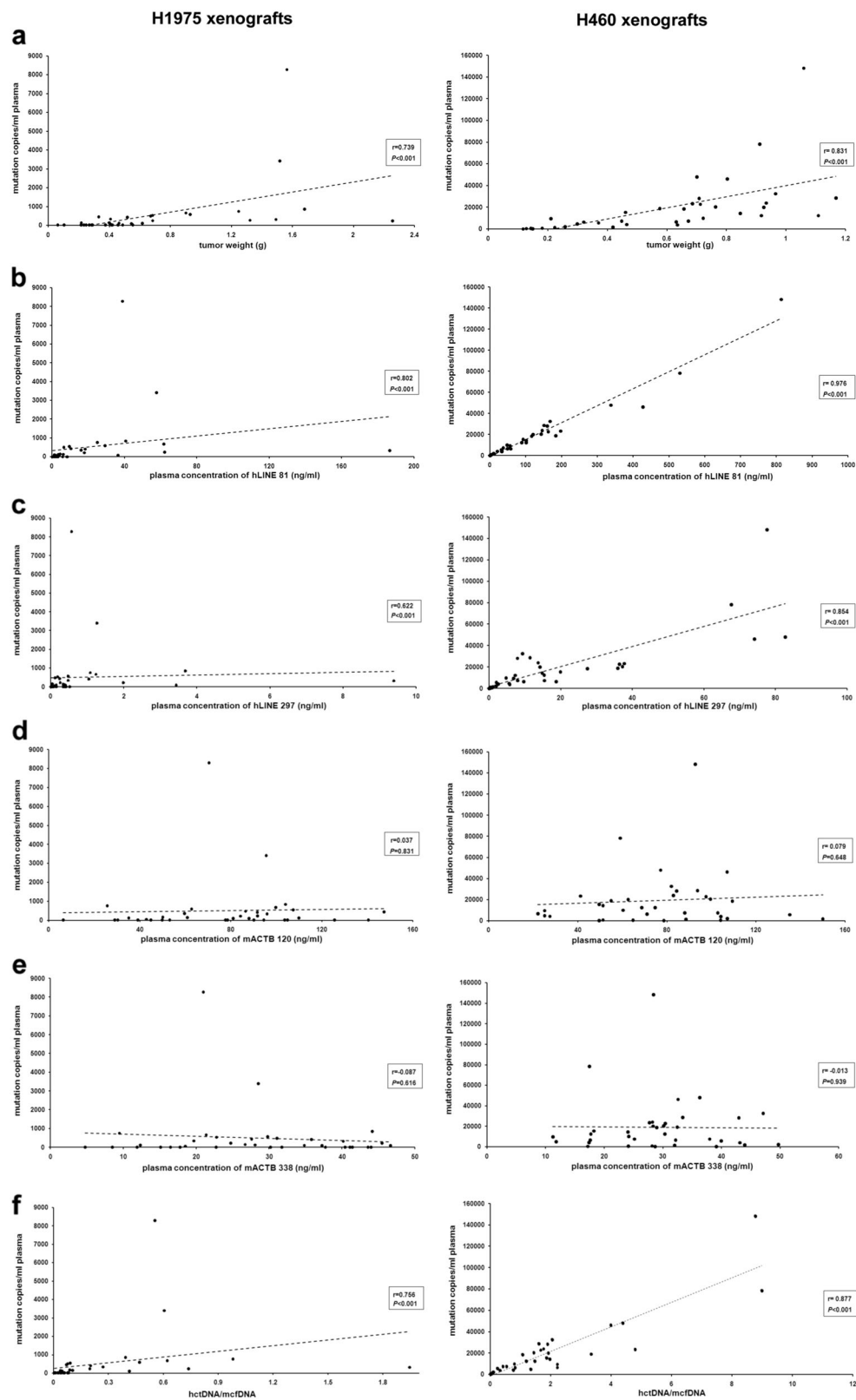


Table 4 demonstrated that only when tumor weight was greater than 0.65 g and 0.2 g in H1975 and H460 xenografted mice, respectively, it was possible to detect gene

mutation of cfDNA. With reference to hctDNA concentration, the present data showed that while they exceeded 9 ng/ml and 3 ng/ml in H1975 and H460 xenografts,

◀ **Fig. 3** Mutation copies/ml plasma relative to tumor weight and plasma hctDNA concentration in xenografted mice. **a** Mutation copies/ml plasma relative to tumor weight. **b** Mutation copies/ml plasma relative to hctDNA concentration obtained with hLINE 81 primer. **c** Mutation copies/ml plasma relative to hctDNA concentration obtained with hLINE 297 primer. **d** Mutation copies/ml plasma relative to mcfDNA concentration obtained with mACTB 120 primer. **e** Mutation copies/ml plasma relative to mcfDNA concentration obtained with mACTB 338 primer. **f** Mutation copies/ml plasma relative to hctDNA/mcfDNA ratio

respectively, it was possible to completely detect plasma cfDNA mutation. In hctDNA groups with amount below 9 ng/ml and 3 ng/ml in H1975 and H460 xenografts, respectively, the positive mutation detection rates were only 9.09% and 0. Furthermore, the ratio of hctDNA/mcfDNA also affected the mutation detection of cfDNA. In H1975 and H460 xenografted mice, the detection rate of positive mutations in hctDNA/mcfDNA groups that were smaller than 0.1 and 0.07, was 13.04% and 0, respectively. These data suggested that hctDNA levels influenced mutation detection and could be a determinant assuring for the feasibility of mutation detection with hctDNA as the analyte.

Discussion

To the best of our knowledge, this is the first study to develop an experimental system for rigorous exploration of the amount of plasma cfDNA necessary for reliable mutation analysis. The results demonstrate that the concentrations of human (tumor)-derived hctDNA, but not mouse-derived mcfDNA, were positively correlated with tumor weight. The fragmentation and detection rates of *EGFR* and *KRAS* gene mutations in plasma cfDNA increased along with hctDNA concentration and tumor weight. When hctDNA concentration reached a certain level, the results of plasma mutation detection were reliable; nonetheless the concentrations of cfDNA that were too low led to a false-negative consequence.

LINE-1, a member of retrotransposon family, is considered as ideal for quantifying human cfDNA. [22–25] The prototypical human *LINE-1* consists of a 5′ untranslated region (5′-UTR), open reading frame (ORF) 1, ORF2, and a 3′-UTR. *hLINE-1* is a kind of highly repetitive sequence with >500,000 copy numbers, widely distributed in human genomes. Because *hLINE-1* family members are divergent at the nucleotide level and display low levels of methylation, they can be used as an ideal test target for increasing the sensitivity of ctDNA quantitative detection. Prak et al. [22] have reported that subset of these copies can be distinguished from mouse orthologues. Primers used in the present study to target *hLINE-1* have been used in previous researches. [23, 24] *ACTB*, a

common reference gene, is widely used in biological analysis. For evaluating mcfDNA content, two primer sets targeting mouse *ACTB* adopted in the present research also referred to the published literature. [13] Higher Q-PCR amplification specificity was confirmed by plasma cfDNA from healthy mice and human individuals, thus ensuring the reliability of our results.

Cell-line derived xenografts are excellent preclinical models for antineoplastic drug development and biomarker discovery. Internal human xenograft models can accurately mimic human cancer disease in defined setting. Nonetheless, the published reports focusing on the use of animal models for studying tumor-derived ctDNA remain scarce. Using xenografts in rats, Garcia-Olmo et al. [26] have shown that ctDNA was detected earlier and more frequently than circulating tumor cells during the spread of colorectal carcinoma. The present study demonstrates that in two types of xenografted mice, hctDNA concentrations slightly increased in plasma samples of mice bearing small tumors, but then increased to a much larger extent in samples from mice with large tumors. When tumor weighed up to 0.8 g in H1975 mice and 0.4 g in H460 mice, respectively, the hctDNA concentration increased by over an order of magnitude. Consistent with our results, Thierry et al. [13] and Rago et al. [23] also found a progressive increase in tumor-derived hctDNA that correlated with tumor size. Another clinical study has shown that ctDNA amounts in patients with colorectal cancer at stages III–IV were significantly higher compared to patients with stages I–II patients, which suggested that ctDNA release was associated with tumor burden. [27] In the larger tumors where nutrients, oxygen and neoangiogenesis were not sufficient to maintain cell growth in all parts of the tumors, ctDNA released from high rate of necrotic or apoptotic tumor cells has increased significantly. Interestingly, in the current study, H1975 and H460 xenografts showed varying ctDNA levels at the similar tumor sizes. The concentrations of hctDNA in H460 xenografts were dramatically higher than those in H1975 xenografted mice. Comparable with our observations, Thierry et al. [13] also noted that the ratio of ctDNA level/tumor weight seemed to be lower in SW620 xenografts compared to HT29 xenografts. These results suggest that the amounts of ctDNA released during tumor growth may be specific characteristics of cell line. Each cell line has individual characteristics that affect propensity to release a certain amount of DNA in a given period, such as percentage of cells dying, or time required between two cell divisions. Above reported phenomena may help in clarifying the clinical data for which it is known that the proportion of circulating DNA is affected by tumor stage, grade, location and type. [28].

DII, calculated as the ratio of larger/shorter fragment concentrations, was used to assess cfDNA fragmentation.

Table 3 Relationship among plasma cfDNA mutation status and tumor weight or hctDNA

| Mutation | Number | Tumor weight (g) | hctDNA level (ng/ml) | | hctDNA/ mcfDNA |
|-------------------|--------|---|---|---|---|
| | | | hLINE 81 | hLINE 297 | |
| EGFR T790M | | | | | |
| Negative | 20 | 0.34 ± 0.23 (0.06–0.62) | 2.63 ± 2.88 (0.37–8.66) | 0.10 ± 0.34 (0.01–0.52) | 0.05 ± 0.04 (0.01–0.09) |
| Positive | 16 | 0.92 ± 0.96 (0.33–2.26) ^{***} | 27.33 ± 41.68 (6.35–186.76) ^{***} | 0.81 ± 1.52 (0.12–9.39) ^{***} | 0.41 ± 0.49 (0.07–1.95) ^{***} |
| KRAS Q61H | | | | | |
| Negative | 4 | 0.14 ± 0.03 (0.12–0.15) | 0.83 ± 1.37 (0.37–2.08) | 0.18 ± 0.26 (0.09–0.39) | 0.01 ± 0.02 (0.01–0.03) |
| Positive | 32 | 0.68 ± 0.47 (0.18–1.17) ^{**} | 96.58 ± 127.50 (3.35–813.25) ^{**} | 12.60 ± 28.17 (0.41–82.70) ^{**} | 1.54 ± 1.58 (0.07–8.99) ^{**} |

^{**} $P < 0.01$, ^{***} $P < 0.001$

Table 4 Mutation detection rate between groups classified with tumor weight and hctDNA

| Xenografts mutation | Parameter | Number | Mutation | | Mutation rate (%) |
|------------------------|--------------------------------|--------|----------|--------------------|--------------------|
| | | | Negative | Positive | |
| H1975 EGFR T790M | Tumor weight (g) | | | | |
| | <0.65 | 24 | 20 | 4 | 16.67 |
| | ≥0.65 | 12 | 0 | 12 | 100 ^{***} |
| | hctDNA level (hLINE 81, ng/ml) | | | | |
| | <9 | 22 | 20 | 2 | 9.09 |
| | ≥9 | 14 | 0 | 14 | 100 ^{***} |
| H460 KRAS Q61H | hctDNA/mcfDNA | | | | |
| | <0.1 | 23 | 20 | 3 | 13.04 |
| | ≥0.1 | 13 | 0 | 13 | 100 ^{***} |
| | Tumor weight (g) | | | | |
| | <0.2 | 5 | 4 | 1 | 20.00 |
| | ≥0.2 | 31 | 0 | 31 | 100 ^{***} |
| | hctDNA level (hLINE 81, ng/ml) | | | | |
| | <3 | 4 | 4 | 0 | 0 |
| | ≥3 | 32 | 0 | 32 | 100 ^{***} |
| | hctDNA/mcfDNA | | | | |
| <0.07 | 4 | 4 | 0 | 0 | |
| ≥0.07 | 32 | 0 | 32 | 100 ^{***} | |

^{***} $P < 0.001$

The present study showed that hDII was smaller than mDII and that it decreased significantly along with tumor size enlargement. This suggests that hctDNA fragmentation degree was more obvious in xenografted mice bearing larger tumor burdens. However, mDII was not correlated with tumor weight. Ellinger et al. [29] have shown that DII (384/106 bp ratio) was 0.348 and 0.681 in 74 patients with

testicular cancer and 35 healthy volunteers, respectively. In addition, they have shown that high fragmentation of ctDNA in patients with bladder cancer correlated with mortality, indicating that ctDNA fragmentation degree may reveal a poor prognosis. [30] Similarly, the prognosis for patients with breast cancer whose DII was smaller than median were also poor. [25] In this study, we also found that compared with hctDNA concentration quantified with hLINE 81 primer (81 bp amplicon), the corresponding hctDNA content obtained using hLINE 297 primer set (297 bp amplicon) was significantly lower. We speculated that this may be related to the fragment deriving from tumor ctDNA approximately distributed less than 200 bp, further verifying that hctDNA is different from genomic DNA fragment length. Consistent with our results, Mouliere et al. [31] have compared ctDNA content using three human KRAS primer pairs that amplify sequences of 73 bp, 145 bp and 300 bp. They showed that the percentage of tumor-derived ctDNA in the range of 60–100 bp was very high (71%) and that it decreased sharply to 2% for fragments larger than 400 bp.

The ddPCR method, which is a highly sensitive technology for mutation identification, can render a DNA template scattered in about 20,000 droplets reaction systems and can detect each amplification reaction. The sensitivity of the ddPCR assay of 0.01% is superior to Amplification Refractory Mutation System (ARMS) approach of 0.1%. [32] The advantages of ddPCR are as follows: true absolute quantification, higher accuracy, resolution and sensitivity. In the present study, using this approach, the mutation status of *EGFR* T790M and *KRAS* Q61H were evaluated in plasma cfDNA of xenografted mice. Our results showed that the number of mutated gene copies in 1 ml plasma was positively correlated with tumor weight. In H1975 xenografts, when tumor weight was less than 400 mg, the mutant copy number in plasma was fairly low. However, when tumor weight exceeded 1,500 mg, the number of mutation

copies increased to the maximum of 8280 copies/ml in plasma. Consistent with our findings, Zhu et al. [33] have reported that quantitative cell-free circulating *EGFR* mutation concentration by ddPCR analysis is correlated with tumor burden in advanced NSCLC patients. Ohira et al. [34] have demonstrated that tumor volume was significantly higher in the cfDNA mutation-positive patients than in the negative patients ($159.1 \pm 58.0 \text{ cm}^3$ vs. $52.5 \pm 9.9 \text{ cm}^3$, $P = 0.014$), suggesting that tumor volume is a determinant of the feasibility of the analyte. Our mutation detection data show that all samples in our data were divided into two groups: the positive group with detectable mutations and negative group without detectable mutations. Our results indicated that the mutation status of cfDNA in smaller tumors was mostly negative, or vice versa. Subsequently, in order to explore the correlation between mutation detection accuracy and tumor weight, the positive mutation detection rate was compared between different tumors classified by the cutoff of 0.65 g and 0.2 g in H1975 and H460 mice, respectively. This study showed that mutation-positive rates (both 100% in H1975 and H460 mice) in larger tumors were significantly higher compared to that (16.67% and 20% in H1975 and H460 mice, respectively) in smaller tumors. The level of cfDNA was not correlated with mutation detection status. On the contrary, htDNA concentrations were positively correlated with the number of mutation copies of the gene in 1 ml plasma. The mutation tests were mostly negative when htDNA concentration was low. With a specific cutoff value of htDNA concentration, the mutation detection rates in higher htDNA groups were both 100% in H1975 and H460 mice. However, in lower htDNA groups, they were only 9.09% and 0, respectively. The idea that lower ctDNA amounts may affect mutation detection suggested that negative mutation of cfDNA in clinical test may be false negative due to the low concentration of ctDNA. Studies have shown that chemotherapy promoted the release of ctDNA. Swystun et al. [35] have reported that patients receiving chemotherapy to treat breast cancer had elevated levels of cfDNA 24 h post-chemotherapy. A similar phenomenon was also observed in patients with metastatic colorectal cancer, where their ctDNA content potentiated to small peaks on the third day after initial chemotherapy and then decreased. [36] In clinical practice, blood collected at the peak stage of ctDNA released from tumor cells in patients receiving chemotherapy could improve the reliability of mutation detection by liquid biopsy.

In conclusion, the present study suggests that ctDNA concentrations can be useful for reliable mutation detection by analysis of cfDNA. When ctDNA content reaches a specific level, the results can be used as evaluation indicators for targeted therapy. The greatest benefits of cfDNA can be obtained alongside the highly sensitive ddPCR and

next-generation sequencing (NGS) methods for blood testing.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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