



Blood digital polymerase chain reaction as a potential method to detect human epidermal growth factor receptor 2 amplification in non-small cell lung cancer

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Background: This study aimed to verify the feasibility of human epidermal growth factor receptor-2 (*HER2*) amplification detection by digital polymerase chain reaction (dPCR) in non-small cell lung cancer (NSCLC) patients and explore whether *HER2* amplification could be detected in circulating tumor DNA (ctDNA) by dPCR.

Methods: A total of 112 fresh biopsy tissues and 88 blood samples from NSCLC patients were collected. The serum ctDNA was obtained from blood samples. The copy number of the *HER2* gene was evaluated by dPCR and next-generation sequencing (NGS). The sensitivity/specificity and survival analysis were performed by the receiver operating characteristic (ROC) curve. The survival analysis was performed by Kaplan-Meier (KM) curve and univariate Cox regression analysis was also conducted.

Results: ROC analysis showed a good prediction result for *HER2* amplification in blood samples by dPCR. The survival analysis showed that the median overall survival (OS) in the *HER2* negative group detected by blood dPCR was significantly different from the positive group. The results of multivariate Cox regression were the same as those of survival analysis.

Conclusions: Blood dPCR might be a potential method to detect *HER2* amplification in NSCLC. Amplification of the *HER2* gene detected by dPCR was correlated with OS in NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); human epidermal growth factor receptor 2 (*HER2*); digital polymerase chain reaction (dPCR); circulating tumor DNA (ctDNA); overall survival (OS)

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Introduction

Lung cancer is one of the most lethal cancers and is a serious threat to human life (1). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancers (2), and the 5-year survival rate among all NSCLC patients is about 10% (3). Thus, it is necessary to improve the clinical diagnosis and prognosis of NSCLC.

Human epidermal growth factor receptor 2 (*HER2*) is a member of the epidermal growth factor receptor (*EGFR*) subfamily (4) and *HER2* amplification has been detected in NSCLC (5). Previous studies have shown that *HER2* gene amplification was present in 10–20% of NSCLC patients (6,7). Notably, *HER2* amplification rates have been shown to be associated with the drug resistance in NSCLC (8,9). At present, the clinical detection of *HER2* amplification is generally by immunohistochemistry (IHC) or fluorescence *in situ* hybridization (FISH) (10). However, IHC and FISH have some limitations, including difficulty in obtaining adequate samples (11).

Digital polymerase chain reaction (dPCR), a new nucleic acid detection technology, can achieve quantitative analysis of target nucleic acid molecules and accurately analyze the target gene copy number variation in tumor tissue or blood at the nucleic acid level (12). It could make sample dispersed and diluted resulting to statistically one or no DNA molecule in each chamber for further amplification instead of performing amplification in bulk sample as traditional polymerase chain reaction (PCR) (13). Currently, the sample dispersion method could be droplet-based, microwell-base, channel-based, hydrogel-based and printing-based (13). This technique could avoid the signal of rare genetic changes being ignored and largely improving the sensitivity and precision of detecting rare genetic aberrations. Previous studies have demonstrated that the accuracy of this technique to detected *EGFR* and *KRAS* mutation through circulating tumor DNA (ctDNA) in NSCLC (14,15). However, whether this technique could detect the amplification of *HER2* in NSCLC by plasma genotyping had not been discussed previously. Besides dPCR, next-generation sequencing (NGS) is also used to reveal *HER2* alterations in NSCLC patients (16). However, compared with NGS, the validity of dPCR, and the feasibility of detecting in blood samples in NSCLC patients are still unclear.

In this study, a comparative analysis of dPCR and NGS was performed regarding their efficacy in *HER2* amplification detection in fresh tissues and blood samples

of NSCLC patients. We verified the feasibility of dPCR detection compared with NGS and explored the possibility of detecting NSCLC *HER2* amplification in blood samples instead of tissue samples. We present the following article in accordance with the STARD reporting checklist (available at <https://dx.doi.org/10.21037/tlcr-21-860>).

Methods

Patients

As we would like to evaluate the efficacy of dPCR in detecting *HER2* amplification through tissue and blood samples, we involved a total of 112 biopsy tissues and 88 blood samples from NSCLC patients who were treated from November 2017 to May 2019 in Shanghai Pulmonary hospital to have relevant test. Clinical information was collected from each participant. Histological diagnoses were made according to the WHO classification (17), and the stages were classed according to the International Association for the Study of Lung Cancer (IASLC) 8th edition (18). Patients who were diagnosed as NSCLC and older than 18 years old could be involved. Written consent was provided by all participants. The study was approved by the Ethics Committee of Shanghai Pulmonary Hospital (No. K20-275). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The patients' characteristics would be blinded for researchers who performed the test.

Extraction of serum ctDNA

The ctDNA of blood samples was extracted using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany).

DNA extraction from tissue sample

DNA was extracted from fresh tissue samples preserved in RNAlater. DNA extraction was performed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's recommendation.

HER2 gene copy number evaluation by dPCR

The copy number variation of the *HER2* gene in the genomic DNA (gDNA) of all tissues and ctDNA of blood samples was detected by dPCR. Based on Beacon

Designer™ 8.12 software (Premier Biosoft, Palo Alto, CA, USA) and the human *HER2* gene sequence, the sequence-specific oligonucleotide primer and TaqMan probe were designed to detect *HER2* and elongation factor Tu GTP binding domain containing 2 (*EFTUD2*) of the internal reference gene. All the primers were synthesized by Shanghai Biotechnology Co., Ltd. (Shanghai, China). The cycling conditions were the same as previously described (19). The sequence of primers were listed below: *HER2*-F: 5'-CTGCGGATTGTGCGAGG-3'; *HER2*-R: 5'-CAGCGGGTCTCCATTGTC-3'; *HER2*-probe: 5'-CCCAGCTCTTTGAGGACAAC-3'; *EFTUD2*-F: 5'-CTCTTCAATATCATGGACACTCCAG-3'; *EFTUD2*-R: 5'-CGCAAAACCAAGACAAGGTTTC-3'; *EFTUD2*-probe: 5'-GGACATCCTTTGGCTTTTGA-3' (Table S1).

HER2 gene copy number evaluation by NGS

The copy number variation of the *HER2* gene in the gDNA of all tissue samples and ctDNA of blood samples was detected by NGS. The gDNA with a double terminal 8-base UDI connector and ctDNA with a single terminal 8-base plus 8-base UMI internal connector was used for the library construction, respectively. Then, the library was hybridized with an Agilent SureSelect^{QXT} reagent kit and blocking agent (Agilent Technologies, Santa Clara, CA, USA). Finally, PCR was used to enrich the captured target bands, and the library was prepared after the capture. After fragment size quantitation by Qubit 4.0 (Thermo Fisher, Waltham, MA, USA) concentration and 4200 Bioanalyzer (Agilent), the library was quantified by quantitative polymerase chain reaction (qPCR), mixed, and then sequenced on NovaSeq 6000 platform (Illumina, San Diego, CA, USA; 4 g tissue data, 15 g blood data). The original data obtained after sequencing was automatically converted into FASTQ data using bcl2fastq software (Illumina) for subsequent data analysis.

The optimal cutoff value for dPCR

The cutoff value of dPCR was determined according to the NGS result. For the tissue samples, when the amplification frequency of *HER2* detected by dPCR was $\geq 61.38\%$ (amplification multiple ≥ 1.59 times), it was consistent with that detected by NGS (amplification multiple ≥ 1.42). For blood (plasma) samples, when the amplification frequency of *HER2* detected by dPCR was $\geq 58.81\%$ (amplification

multiple ≥ 1.43 times), it was consistent with that detected by NGS (amplification multiple ≥ 1.45 times).

Statistical analyses

Data were analyzed using the software SAS 9.0 (SAS Institute, Cary, NC, USA). Indeterminate result of test would not be included for further analysis. All data were represented as the mean \pm standard deviation (SD). Student's *t*-test was used to analyze continuous variables, and chi-square tests were used to analyze categorical variables. Moreover, the survival analysis was performed based on the Kaplan-Meier (KM) method. The log-rank test was used to calculate statistical differences in survival status.

Furthermore, the sensitivity and specificity analysis of each method was performed based on the ROC curve. The Cox proportional hazards model was used in the regression analysis. A P value < 0.05 was considered statistically significant.

Results

Participant characteristics

We recruited 112 participants (59 male; 53 female) to the tissue sample group and detected *HER2* with dPCR. Among them, 36 were smokers and 76 were non-smokers. A total of 63 participants had *EGFR* mutations, and 51 had received at least first-line therapy with tyrosine kinase inhibitors (TKIs) (Table 1).

A total of 90 participants in the tissue sample group were also detected by NGS (46 male; 44 female). A total of 29 were smokers, 61 were non-smokers; 12 were squamous and 78 non-squamous; 52 participants had *EGFR* mutations, and 42 had received at least first-line therapy with TKIs (Table 2).

A total of 88 participants (46 male; 42 female) were enrolled and had *HER2* detected by dPCR in their blood samples. Among them, 32 were smokers and 56 were non-smokers (Table 3). Sixteen blood samples were also analyzed by NGS and patients' characteristics were summarized in Table 4.

The sensitivity and specificity analysis based on ROC

The ROC analysis for the tissue sample (or blood samples) detected by dPCR (or NGS) is shown in Figure 1.

The results showed that the area under the curve (AUC) for tissue dPCR was 0.533 [95% confidence interval (CI):

Table 1 NSCLC patient characteristics according to *HER2* expression and amplification detected by dPCR in tissue samples

Factors	Negative group (n=63), n (%)	Positive group (n=49), n (%)	χ^2	P value
Sex			0.205	0.651
Male	32 (50.8)	27 (55.1)		
Female	31 (49.2)	22 (44.9)		
Age (years)			0.291	0.590
<60	21 (33.3)	14 (28.6)		
≥60	42 (66.7)	35 (71.4)		
Smoking			1.757	0.185
No	46 (73.0)	30 (61.2)		
Yes	17 (27.0)	19 (38.8)		
NSCLC types			9.253	0.010
ac-NSCLC	45 (71.4)	28 (57.1)		
sq-NSCLC	3 (4.8)	12 (24.5)		
NSCLC	15 (23.8)	9 (18.4)		
TNM stage			0.054	0.816
III	13 (20.6)	11 (22.4)		
IV	50 (76.4)	38 (77.6)		
Bone metastasis			0.260	0.610
No	44 (69.8)	32 (65.3)		
Yes	19 (30.2)	17 (34.7)		
Pulmonary metastasis			1.418	0.234
No	41 (65.1)	37 (75.5)		
Yes	22 (34.9)	12 (24.5)		
Brain metastases			0.139	0.709
No	51 (81.0)	41 (83.7)		
Yes	12 (19.0)	8 (16.3)		
Pleura metastases			0.361	0.548
No	43 (68.3)	36 (73.5)		
Yes	20 (31.7)	13 (26.5)		
Adrenal gland metastases			2.885	0.089
No	62 (98.4)	45 (91.8)		
Yes	1 (1.6)	4 (8.2)		
Liver metastases			0.138	0.710
No	58 (92.1)	46 (93.9)		
Yes	5 (7.9)	3 (6.1)		

Table 1 (continued)

Table 1 (continued)

Factors	Negative group (n=63), n (%)	Positive group (n=49), n (%)	χ^2	P value
LN metastases			0.065	0.798
No	61 (96.8)	47 (95.9)		
Yes	2 (3.2)	2 (4.1)		
EGFR mutation			0.888	0.642
Common	34 (79.1)	16 (69.6)		
Uncommon	7 (16.3)	6 (26.1)		
WT	2 (4.7)	1 (4.3)		
TKI			2.776	0.096
No	21 (38.9)	23 (56.1)		
Yes	33 (61.1)	18 (43.9)		

P<0.05 was considered as significant different. NSCLC, non-small cell lung cancer; HER2, human epidermal growth factor receptor-2; dPCR, digital polymerase chain reaction; ac-NSCLC, lung adenocarcinoma; sq-NSCLC, lung squamous cell carcinoma; TNM, tumor, lymph node and metastasis; LN, Lymph node; EGFR, epidermal growth factor receptor; WT, wide type; TKI, Tyrosine kinase inhibitors.

Table 2 NSCLC patient characteristics according to *HER2* expression and amplification detected by NGS in tissue samples

Factors	Negative group (n=44), n (%)	Positive group (n=46), n (%)	χ^2	P value
Sex			2.166	0.141
Male	19 (43.2)	27 (58.7)		
Female	25 (56.8)	19 (41.3)		
Age (years)			2.233	0.136
<60	18 (40.9)	12 (26.1)		
≥60	26 (59.1)	34 (73.9)		
Smoking			2.056	0.152
No	33 (75.0)	28 (60.9)		
Yes	11 (25.0)	18 (39.1)		
NSCLC types			5.570	0.062
ac-NSCLC	33 (75.0)	24 (52.2)		
sq-NSCLC	3 (6.8)	9 (19.6)		
NSCLC	8 (18.2)	13 (28.3)		
TNM stage			1.985	0.159
III	7 (15.9)	13 (28.3)		
IV	37 (84.1)	33 (71.7)		
Bone metastasis			5.852	0.016
No	25 (56.8)	37 (80.4)		
Yes	19 (43.2)	9 (19.6)		

Table 2 (continued)

Table 2 (continued)

Factors	Negative group (n=44), n (%)	Positive group (n=46), n (%)	χ^2	P value
Pulmonary metastasis			0.357	0.550
No	29 (65.9)	33 (71.7)		
Yes	15 (34.1)	13 (28.3)		
Brain metastases			0.028	0.867
No	36 (81.8)	37 (80.4)		
Yes	8 (18.2)	9 (19.6)		
Pleura metastases			1.660	0.198
No	28 (63.6)	35 (76.1)		
Yes	16 (36.4)	11 (23.9)		
Adrenal gland metastases			0.399	0.528
No	42 (95.5)	45 (97.8)		
Yes	2 (4.5)	1 (2.2)		
Liver metastases			3.296	0.069
No	39 (88.6)	45 (97.8)		
Yes	5 (11.4)	1 (2.2)		
LN metastases			1.188	0.276
No	41 (93.2)	45 (97.8)		
Yes	3 (6.8)	1 (2.2)		
EGFR mutation			5.597	0.061
Common	26 (81.3)	17 (73.9)		
Uncommon	3 (9.4)	6 (26.1)		
WT	3 (9.4)	0 (0.0)		
TKI			0.315	0.574
No	20 (47.6)	14 (41.2)		
Yes	22 (52.4)	20 (58.8)		

P<0.05 was considered as significant different. NSCLC, non-small cell lung cancer; HER2, human epidermal growth factor receptor-2; dPCR, digital polymerase chain reaction; ac-NSCLC, lung adenocarcinoma; sq-NSCLC, lung squamous cell carcinoma; TNM, tumor, lymph node and metastasis; LN, Lymph node; EGFR, epidermal growth factor receptor; WT, wide type; TKI, Tyrosine kinase inhibitors.

0.408 to 0.657; P=0.611] with a sensitivity of 55.2% and a specificity of 57.4% (Figure 1A). The AUC for tissue NGS was 0.556 (95% CI: 0.416 to 0.697; P=0.425) with a sensitivity of 68.0% and a specificity of 50.9% (Figure 1B).

The results showed that the AUC for blood dPCR was 0.669 (95% CI: 0.538 to 0.800; P=0.017) with a sensitivity of 80.0% and a specificity of 55.8% (Figure 1C). Meanwhile, the AUC for blood NGS was 0.592 (95% CI: 0.279 to 0.905; P=0.565) with a sensitivity of 42.9% and a specificity of

85.7% (Figure 1D). The P values in blood dPCR were less than 0.05, indicating a good prediction result.

HER2 amplification in tissue samples and blood samples

When *HER2* amplification of tissue samples was ≥ 1.59 times and *HER2* amplification of blood samples ≥ 1.42 times, the results of paired detection of tissue and blood were consistent: 2/3 =66.7%. When the amplification of *HER2* in

Table 3 NSCLC patient characteristics according to *HER2* expression and amplification detected by dPCR in blood samples

Factors	Negative group (n=42), n (%)	Positive group (n=46), n (%)	χ^2	P value
Sex			0.697	0.404
Male	20 (47.6)	26 (56.5)		
Female	22 (52.4)	20 (43.5)		
Age (years)			0.573	0.449
<60	16 (38.1)	14 (30.4)		
≥60	26 (61.9)	32 (69.6)		
Smoking			0.015	0.904
No	27 (64.3)	29 (63.0)		
Yes	15 (35.7)	17 (37.0)		
NSCLC types			0.441	0.802
ac-NSCLC	27 (64.3)	29 (63.0)		
sq-NSCLC	7 (16.7)	6 (13.0)		
NSCLC	8 (19.0)	11 (23.9)		
TNM stage			1.004	0.316
III	11 (26.2)	8 (17.4)		
IV	31 (73.8)	38 (82.6)		
Bone metastasis			0.554	0.457
No	28 (66.7)	34 (73.9)		
Yes	14 (33.3)	12 (26.1)		
Pulmonary metastasis			0.003	0.958
No	29 (69.0)	32 (69.6)		
Yes	13 (31.0)	14 (30.4)		
Brain metastases			0.709	0.400
No	35 (83.3)	35 (86.1)		
Yes	7 (16.7)	11 (23.9)		
Pleura metastases			0.047	0.828
No	31 (73.8)	33 (71.7)		
Yes	11 (26.2)	13 (28.3)		
Adrenal gland metastases			0.128	0.721
No	40 (95.2)	43 (93.5)		
Yes	2 (4.8)	3 (6.5)		
Liver metastases			1.758	0.185
No	41 (97.6)	42 (91.3)		
Yes	1 (2.4)	4 (8.7)		

Table 3 (continued)

Table 3 (continued)

Factors	Negative group (n=42), n (%)	Positive group (n=46), n (%)	χ^2	P value
LN metastases			0.264	0.608
No	41 (97.6)	44 (95.7)		
Yes	1 (2.4)	2 (4.3)		
EGFR mutation			2.557	0.278
Common	17 (70.8)	21 (80.8)		
Uncommon	7 (29.2)	4 (15.4)		
WT	0 (0.0)	1 (3.8)		
TKI			0.001	0.979
No	16 (47.1)	18 (47.4)		
Yes	18 (52.9)	20 (52.6)		

$P < 0.05$ was considered as significant different. NSCLC, non-small cell lung cancer; HER2, human epidermal growth factor receptor-2; dPCR, digital polymerase chain reaction; ac-NSCLC, lung adenocarcinoma; sq-NSCLC, lung squamous cell carcinoma; TNM, tumor, lymph node and metastasis; LN, lymph node; EGFR, epidermal growth factor receptor; WT, wide type; TKI, tyrosine kinase inhibitors.

Table 4 NSCLC patient characteristics according to *HER2* expression and amplification detected by NGS in blood samples

Factors	Negative group (n=13), n (%)	Positive group (n=3), n (%)	χ^2	P value
Sex			0.028	0.868
Male	5 (38.5)	1 (33.3)		
Female	8 (61.5)	2 (66.7)		
Age (years)			0.417	0.519
<60	7 (53.8)	1 (33.3)		
≥ 60	6 (46.2)	2 (66.7)		
Smoking			0.461	0.497
No	11 (84.6)	2 (66.7)		
Yes	2 (15.4)	1 (33.3)		
NSCLC types			0.894	0.344
ac-NSCLC	11 (84.6)	3 (100.0)		
NSCLC	2 (15.4)	0 (0.0)		
TNM stage			0.430	0.512
III	1 (7.7)	0 (0.0)		
IV	12 (92.3)	3 (100.0)		
Bone metastasis			3.225	0.073
No	7 (53.8)	3 (100.0)		
Yes	6 (46.2)	0 (0.0)		

Table 4 (continued)

Table 4 (continued)

Factors	Negative group (n=13), n (%)	Positive group (n=3), n (%)	χ^2	P value
Pulmonary metastasis			3.013	0.083
No	11 (84.6)	1 (33.3)		
Yes	2 (15.4)	2 (66.7)		
Brain metastases			0.130	0.718
No	10 (76.9)	2 (66.7)		
Yes	3 (23.1)	1 (33.3)		
Pleura metastases			3.225	0.073
No	7 (53.8)	3 (100.0)		
Yes	6 (46.2)	0 (0.0)		
Adrenal gland metastases			–	–
No	13 (100.0)	3 (100.0)		
Yes	0 (0.0)	0 (0.0)		
Liver metastases			0.461	0.497
No	11 (84.6)	2 (66.7)		
Yes	2 (15.4)	1 (33.3)		
LN metastases			3.662	0.056
No	13 (100.0)	2 (66.7)		
Yes	0 (0.0)	1 (33.3)		
EGFR mutation			–	–
Common	10 (100.0)	1 (100.0)		
Uncommon	0 (0.0)	0 (0.0)		
WT	0 (0.0)	0 (0.0)		
TKI			0.200	0.655
No	4 (33.3)	1 (50.0)		
Yes	8 (66.7)	1 (50.0)		

P<0.05 was considered as significant different. NSCLC, non-small cell lung cancer; HER2, human epidermal growth factor receptor-2; NGS, next generation sequencing; ac-NSCLC, lung adenocarcinoma; TNM, tumor, lymph node and metastasis; LN, lymph node; EGFR, epidermal growth factor receptor; WT, wide type; TKI, tyrosine kinase inhibitors.

tissue samples was <1.59 times and that of *HER2* in blood samples <1.42 times, the results of dPCR match detection on tissue and blood samples were consistent: 96/97 =98.9%.

Correlation between *HER2* amplification results and clinical data

Based on the tissue samples, we analyzed the correlation between clinical factors and *HER2* amplification detected

by dPCR and NGS in NSCLC patients and dPCR analysis showed that pathological type was correlated with *HER2* amplification (P=0.010, Table 1). NGS analysis showed that bone metastasis was associated with *HER2* amplification (P=0.016, Table 2).

Survival analysis

The results of univariate Cox regression analysis indicated

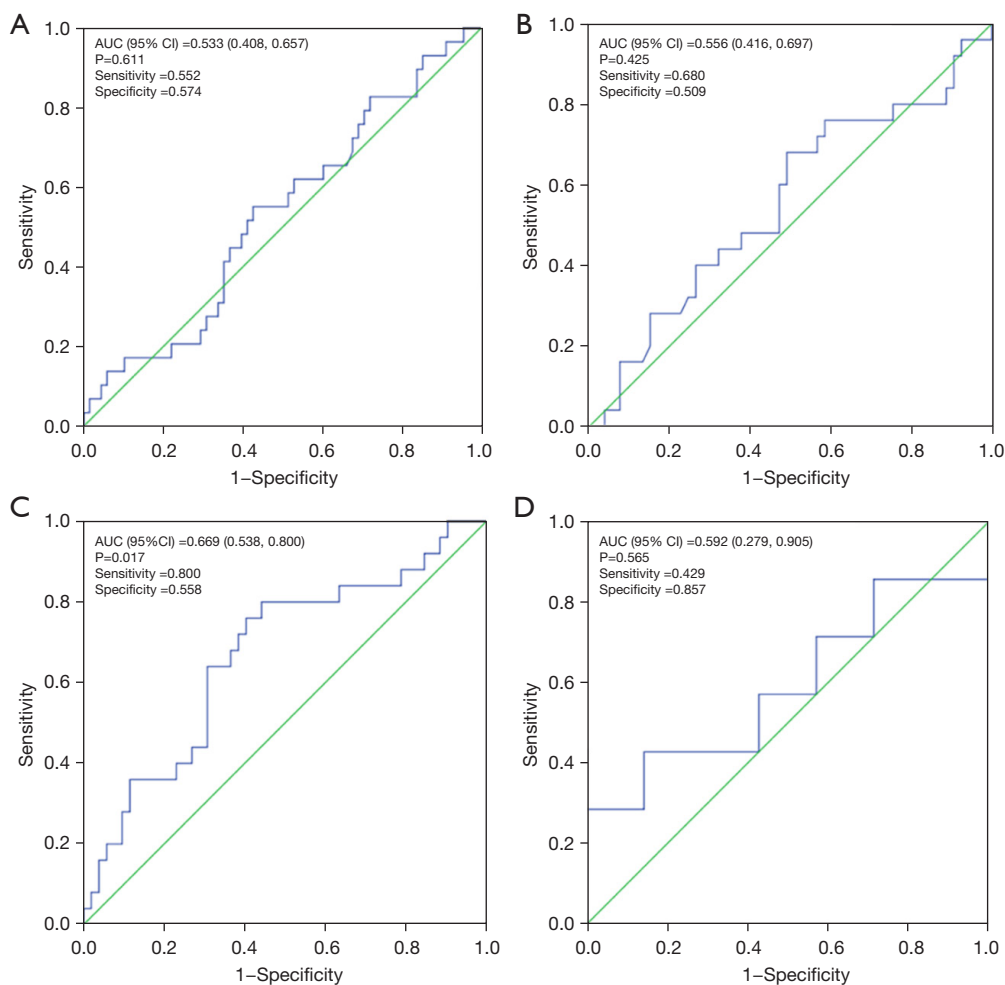


Figure 1 The sensitivity and specificity study based on receiver-operating characteristic (ROC). (A) The ROC analysis for non-small cell lung cancer (NSCLC) tissue samples detected by digital polymerase chain reaction (dPCR): the result showed that the area under the curve (AUC) for tissue dPCR was 0.533 (95% CI: 0.408–0.657; $P=0.611$) with a sensitivity of 55.2% and a specificity of 57.4%. (B) The ROC analysis for NSCLC tissue samples detected by next generation sequencing (NGS): the AUC for tissue NGS was 0.556 (95% CI: 0.416–0.697; $P=0.425$) with a sensitivity of 68.0% and a specificity of 50.9%. (C) The ROC analysis for NSCLC blood samples detected by dPCR: the result showed that the AUC for blood dPCR was 0.669 (95% CI: 0.538–0.800; $P=0.017$) with a sensitivity of 80.0% and a specificity of 55.8%. (D) The ROC analysis for NSCLC blood samples detected by NGS. The X-axis represented the specificity, while the Y-axis represented the sensitivity: the AUC for blood NGS was 0.592 (95% CI: 0.279–0.905; $P=0.565$) with a sensitivity of 42.9% and a specificity of 85.7%.

that baseline information such as age ≥ 60 *vs.* <60 , hazard ratio (HR) (95% CI): 4.621 (1.379 to 15.488), $P=0.013$], pleura metastasis [yes *vs.* no, HR (95% CI): 0.274 (0.082 to 0.920), $P=0.036$], and NSCLC type [adenocarcinoma *vs.* squamous cell carcinoma, HR (95% CI): 2.153 (1.361 to 3.405), $P=0.001$] were significantly associated with OS (Table S2). In addition, KM survival analysis showed that the differences between OS and *HER2* amplification

detected by tissue NGS (positive *vs.* negative, HR (95% CI): 55.38 (39.26 to 71.50), $P=0.053$) and blood dPCR (positive *vs.* negative, HR (95% CI): 20.61 (16.95 to 24.26), $P=0.044$).

In tissue dPCR, median OS of the negative group and positive group was 39.00 and 66.01 months, respectively ($P=0.561$) (Figure 2A). In tissue NGS, median survival time of the negative group and the positive group was 39.26

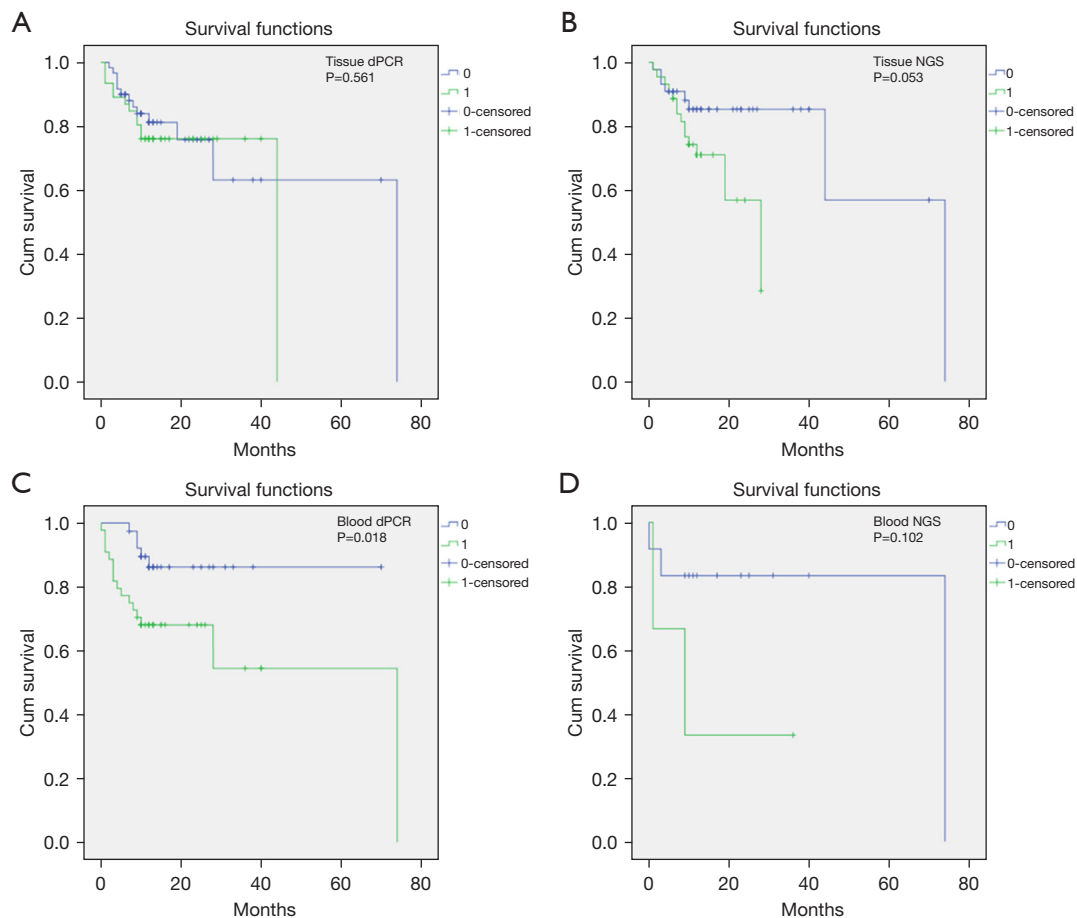


Figure 2 The survival rates for human epidermal growth factor receptor-2 (*HER2*)-negative (blue line) and *HER2*-positive (green line) non-small cell lung cancer (NSCLC) patients in current study. (A) The tissue sample of NSCLC patients detected by digital polymerase chain reaction (dPCR). (B) The tissue sample of NSCLC patients detected by next generation sequencing (NGS). (C) The blood sample of NSCLC patients detected by dPCR. (D) The blood sample of NSCLC patients detected by NGS. The X-axis represented the months, while the Y-axis represented the total survival.

and 71.50 months, respectively, with a marginal difference ($P=0.053$) (Figure 2B). Moreover, in blood dPCR, the median survival time of the negative *HER2* amplification group and the positive group was 61.67 (54.84 to 68.49) months and 45.44 (31.16 to 59.71) months, respectively ($P=0.018$) (Figure 2C). Multivariate Cox regression analysis showed that the risk of death in the blood dPCR *HER2* amplification positive group was significantly higher than that in the negative group [HR (95% CI): 3.874 (1.356 to 11.069), $P=0.011$] (Table S3). Moreover, the multivariate Cox regression analysis of tissue NGS showed that after adjusting for factors such as age, pleura metastasis, NSCLC pathology type, and smoking, *HER2* amplification was not significantly associated with the risk of death (Table S4).

Furthermore, in blood NGS, the median survival time of the negative *HER2* amplification group and the positive group was 61.92 (43.19 to 80.65) months and 15.33 (0 to 32.28), respectively ($P=0.102$) (Figure 2D).

Discussion

As the most common type of lung cancer, the incidence of NSCLC is high (20). Although dPCR is an approach for detecting biomarkers in cancer, whether dPCR is feasible for *HER2* amplification is unknown. In this study, we used both dPCR and NGS to detect *HER2* amplification in the tumor tissues and blood of NSCLC patients. The ROC analysis results showed a good prediction result of

dPCR in detecting *HER2* amplification in blood samples. Furthermore, survival analysis showed that the median survival time was longer in the negative *HER2* amplification group than in the positive group as detected by blood dPCR, which was accordant with the results of multivariate Cox regression. Finally, the amplification multiple in tissue samples was ≥ 1.59 times and amplification multiple in blood samples was ≥ 1.43 times the recommended cutoff values for dPCR detection in patients with NSCLC.

Amplification/overexpression of the *HER2* gene is related to the occurrence and development of tumors (21-23). As for lung adenocarcinoma (LUAD), *HER2* aberration could be detected in about 6% patients with no *EFGR/KRAS/ALK* alteration (24). In LUAD, patients with *HER2* alteration would have higher risk of lung and bone metastases (25). Moreover, *HER2* amplification was a mechanism of resistant to osimertinib (26). *HER2* was also an actionable driver and currently some drugs targeted *HER2* alteration had been tested in clinical trials (27,28). Based on these studies, detecting *HER2* alterations could help us define a novel subset in *EFGR/KRAS/ALK* negative LUAD patients, evaluating the metastatic risk of patients, understanding EGFR-TKI resistance and selecting proper patients for HER2-TKI treatment. Thus, a suitable method for *HER2* gene amplification/mutation detection is necessary for patient classification and cancer intervention (29). To date, the NGS method has been commonly used for *HER2* amplification detection (30). A previous study indicated that NGS can identify the coexistence of *HER2* amplification and mutation in NSCLC (31). However, NGS also has disadvantages; its application is limited by the reading length of 200–500 bp segments (32).

A recent cross-platform comparison study based on ctDNA samples obtained from patients with NSCLC showed that dPCR had a unique advantage in gene mutation detection compared with NGS (33). Researchers have shown the dPCR to be a more precise and less subjective alternative for quantifying *HER2* DNA amplification in cancer (34). A previous study showed that dPCR was sensitive in detecting *EGFR* mutations from ctDNA in advanced NSCLC patients (14). The feasibility of dPCR for the quantitative and dynamic detection of *EGFR* mutations has been supported in comparison with NGS in lung cancer (35). Mehrotra *et al.* indicated that dPCR had 100% sensitivity in mutation detection and also revealed the correlation between OS and gene mutation (36). In our

study, sensitivity and specificity analysis based on the ROC showed an excellent prediction result of dPCR detection on *HER2* amplification. Meanwhile, the survival analysis showed that the median survival time between negative and positive *HER2* DNA amplification groups detected by blood dPCR was significantly different, which was accordant with the result of multivariate Cox regression analysis. Thus, we speculated that dPCR could be applied in detecting *HER2* amplification of NSCLC. And the *HER2* amplification detected by dPCR also could be a potential method for predicting the OS of NSCLC patients.

The continuous research of targeted and immunotherapy drugs has resulted in the increase of treatment opportunities for NSCLC (37); however, it is challenging to collect tissue samples from lung cancer patients (38). The measurement of mutations in blood ctDNA may transform the management of cancer patient (39). It has been shown that dPCR is a useful method with high sensitivity and specificity for detecting *EGFR* mutation in plasma (40). A previous study showed that repeated measures on the same blood sample indicated that dPCR was less variable than another qPCR method (41). Li *et al.* indicated that dPCR improved *EGFR* mutation detection in the liquid but not tissue samples of NSCLC patients (42). In this study, we found that dPCR and NGS had identical efficiency in blood sample detection.

Meanwhile, sensitivity and specificity analysis based on the ROC showed that the P value in blood dPCR was below 0.05. This result demonstrated a good prediction result of dPCR detection on *HER2* amplification in blood samples. Thus, we speculated that it was possible to use blood samples to detect *HER2* amplification in NSCLC. However, there were still some limitations in the current study, due to its small sample size and retrospective study. Thus, further research based on a large sample size prospective study is needed to verify our findings.

In conclusion, dPCR detection of *HER2* gene amplification might be a potential method to predict the OS of NSCLC. Blood samples could be used to detect *HER2* amplification by dPCR.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at <https://dx.doi.org/10.21037/tlcr-21-860>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/tlcr-21-860>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tlcr-21-860>). HV and JX report that they are from Alphamab Oncology Ltd. JW and QZ report that they are from Genetron Health (Beijing) Co.,Ltd. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethic committee of Shanghai Pulmonary Hospital (No. K20-275) and informed consent was taken from all the patients.

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Table S1 The sequence of used primers

Primer name	Sequence or modification
<i>HER2-F</i>	5'-CTGCGGATTGTGCGAGG-3'
<i>HER2-R</i>	5'-CAGCGGGTCTCCATTGTC-3'
<i>HER2-probe</i>	5'-CCCAGCTCTTTGAGGACAAC-3'
<i>HER2-modification</i>	5'-6-FAM, 3'-MGB
<i>EFTUD2-F</i>	5'-CTCTTCAATATCATGGACACTCCAG-3'
<i>EFTUD2-R</i>	5'-CGCAAAACCAAGACAAGTTTC-3'
<i>EFTUD2-probe</i>	5'-GGACATCCTTTGGCTTTTGA-3'
<i>EFTUD2-modification</i>	5'-VIC, 3'-MGB

Table S2 The result of univariate Cox regression analysis

Factors	HR (95% CI)	P value
Age (≥ 60 vs. < 60 years)	4.621 (1.379, 15.488)	0.013
Sex (male vs. female)	0.552 (0.244, 1.249)	0.154
Smoking (yes vs. no)	2.598 (1.182, 5.707)	0.017
NSCLC types (CT: ac-NSCLC)	2.153 (1.361, 3.405)	0.001
TNM stage (III vs. IV)	0.693 (0.273, 1.760)	0.441
Bone metastasis (yes vs. no)	0.683 (0.272, 1.712)	0.416
Pulmonary metastasis (yes vs. no)	0.594 (0.236, 1.494)	0.268
Brain metastases (yes vs. no)	1.023 (0.383, 2.729)	0.964
Pleura metastases (yes vs. no)	0.274 (0.082, 0.920)	0.036
Adrenal gland metastases (yes vs. no)	1.921 (0.451, 8.185)	0.377
Liver metastases (yes vs. no)	0.651 (0.088, 4.824)	0.674
LN metastases (yes vs. no)	1.519 (0.348, 6.630)	0.578
EGFR mutation (uncommon vs. common)	1.390 (0.439, 4.402)	0.575
TKI treatment (yes vs. no)	0.640 (0.231, 1.776)	0.392
Tissue dPCR (positive vs. negative)	1.266 (0.568, 2.824)	0.564
Tissue NGS (positive vs. negative)	2.614 (1.010, 6.765)	0.048
Blood dPCR (positive vs. negative)	3.168 (1.150, 8.725)	0.026
Blood NGS (positive vs. negative)	4.481 (0.627, 32.023)	0.135

$P < 0.05$ was considered as significant different. HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer; CT, control group; ac-NSCLC, lung adenocarcinoma; TNM, the tumor, node, metastases; LN, Lymph node; EGFR, epidermal growth factor receptor; TKI, Tyrosine kinase inhibitors; dPCR, digital polymerase chain reaction; NGS, next generation sequence.

Table S3 The result of multivariate regression analysis for blood samples

Factors	HR (95% CI)	P value
Age (≥ 60 vs. < 60 years)	3.749 (1.011, 13.898)	0.048
Smoking (yes vs. no)	1.274 (0.464, 3.497)	0.639
NSCLC types (CT: ac-NSCLC)	1.480 (0.853, 2.567)	0.164
Pleura metastases (yes vs. no)	0.229 (0.052, 1.005)	0.051
Blood dPCR (positive vs. negative)	3.874 (1.356, 11.069)	0.011

P<0.05 was considered as significant different. HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer; CT, control group; ac-NSCLC, lung adenocarcinoma; dPCR, digital polymerase chain reaction.

Table S4 The result of multivariate regression analysis for tissue samples

Factors	HR (95% CI)	P value
Age (≥ 60 vs. < 60 years)	4.741 (1.058, 21.250)	0.042
Smoking (yes vs. no)	1.446 (0.555, 3.764)	0.450
NSCLC types (CT: ac-NSCLC)	1.674 (0.953, 2.940)	0.073
Pleura metastases (yes vs. no)	0.369 (0.100, 1.355)	0.133
Tissue NGS (positive vs. negative)	1.512 (0.553, 4.136)	0.421

P<0.05 was considered as significant different. HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer; CT, control group; ac-NSCLC, lung adenocarcinoma; NGS, next generation sequence.