Intratumor Heterogeneity of EGFR Activating Mutations in Advanced NSCLC Patients at Single-Cell Level

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Abstract: Introduction: Present study is to explore the controversial problem whether the intratumoral heterogeneity of *EGFR* activating mutation exists at the level of single cancer cells. **Materials and Methods**: Single H1975 cells harbored *EGFR* exon 21 L858R mutation were isolated by flow cytometry to assess the feasibility of single-cell analysis for *EGFR* exon 21 by nested polymerase chain reaction and derect sequence. Then, six patients diagnosed with lung adenocarcinoma whose fresh frozen specimens harbored *EGFR* exon 21 mutation tested by direct sequencing were chosen. All of them received gefitnib treatment and the PFS of three patients was longer than 12 months (Group A) while the PFS of other three patients was shorter than 6 months (Group B). By using the established method based on single H1975 cells, *EGFR* exon 21 mutational status was analyzed in single tumor cells which were captured from tumor sample by Laser Capture Microdissection. **Results:** A total of 104 individual H1975 cells were obtained. The amplification rate and allele drop-out rate were 96.2% and 7.0%, respectively. A total of 135 tumor cells from six patients' samples were captured. The amplification rate of nested PCR was 84.3% (59/70) in Group A and 93.8% (61/65) in Group B (P=0.077). The mutational rate was 86.4% in Group A, which was significantly higher than the total mutational rate 68.9% in Group B (P=0.021).**Conclusion**: The intratumoral heterogeneity of *EGFR* activating mutation in lung adenocarcinoma does exist based on the analysis of single cancer cells.

1. Introduction

Lung cancer has become a leading cause of cancer-related death all over the world[1]. In recent years, several large randomized controlled clinical studies consistently demonstrated that epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) have shown great efficacy in the treatment of non-small cell lung cancer (NSCLC) patients with *EGFR* activating mutation compared with chemotherapy in first line treatments[2-6]. However, the response to EGFR-TKIs is quite different in *EGFR* mutant patients. Some patients experienced longer progression-free survival (PFS) of more than 1 year, whereas some had PFS of shorter than 6 months. Our previous study showed that the relative *EGFR* mutation abundance in tumor tissues could predict benefit from EGFR-TKI treatments[7]. [However, the study demonstrated EGFR heterogeneity based on the tissue level] To our knowledge, phenotypical and functional heterogeneity among cancer cells that might be associated with the response to therapy has long been proposed and accepted[8]. Recently, driver gene *EGFR* activating mutational heterogeneity, including intratumoral heterogeneity, intertumoral heterogeneity and pre- or post-treatment heterogeneity[9] was considered as a potential cause of diverse response to EGFR-TKIs[10-12]. However, heterogeneity of *EGFR* activating mutation and its effect of EGFR-TKIs still remains divergent viewpoint in NSCLC partients[13-15].

The methods in above studies used for detecting *EGFR* status have been established on complex mixture of cells or tissue level. Therefore, misinterpretation for *EGFR* mutational information might occur due to interference of cell populations. Single-cell analysis was direct and better understanding of genetic characteristics of tumors by flow cytometry (FCM) and Laser Capture Microdissection (LCM)[16]. Single tumor cell analysis might provide a deeper insight into the occurrence of intratumoral *EGFR* activating mutation heterogeneity[17].

In the present study, single H1975 cells which harbors *EGFR* L858R heterozygous mutation in exon 21 were isolated by FCM were to evaluate the feasibility of single-cell analysis for *EGFR*

mutation detection. Based on the method, we investigated the occurrence of intratumoral *EGFR* activating mutational heterogeneity at the level of single tumor cells in lung adenocarcenoma which had *EGFR* 21 exon L858R mutation.

2. Materials and Methods

2.1 Cell culture

NSCLC cell line H1975 has L858R heterozygous mutation in exon 21 of the *EGFR* gene[18]. It was kindly presented by professor Tony S. Mok (Prince of Wales Hospital, Hong Kong) in our study. He purchased H1975 cell line from American Type Culture Collection (ATCC). H1975 cells were cultured on flasks coated with RPMI 1640 containing 10% fetal calf serum and incubated at 37℃ in a humidified atmosphere with 5% CO_2. When the cells grew well to 80%~90% of the flasks, we digested them with the use of trypsinase to prepare single cell suspension for isolation.

2.2 Tissue samples

In this study, six patients with advanced lung adenocarcinoma harbored *EGFR* exon 21 L858R mutation under gefitnib treatment in their late stage were screened (Table 1). They have the following clinical characteristics: They were all treated in Guangdong General Hospital and enough tissue preserved at the tumor tissue biobank of the Guangdong Lung Cancer Institute(GLCI); Pathology of their tumors showed pure adenocarcinoma and *EGFR* L858R mutation in exon 21 by direct sequencing; In their first-line treatment they were all treated with gefitnib. Their progression-free survival (PFS) of EGFR-TKIs was 22, 19, 15, 5, 3, 1 months , respectively. The 3 of patient which PFS longer than 12 months was screened as long-term PFS group, and selected 3 patients which PFS of shorter than 6 months as short-term PFS group. All the patients had written informed consent.

Table 1. The clinicopathologic feature of six patients

Patient no.	Sex	Age	PS	Smoking history	Stage	Tumor	Histology	EGFR status	TKI treatment	PFS	Response to TKIs
3647	male	87	1	1	IV	Lung	Ad	L858R	First-line	19	PR
2715	male	47	2	0	IV	Lung	Ad	L858R	First-line	22	SD
4128	male	42	2	1	IV	Lung	Ad	L858R	First-line	15	PR
3669	male	76	1	1	IV	Lung	Ad	L858R	First-line	5	SD
1813	male	60	2	0	IV	Lung	Ad	L858R	First-line	3	PD
3651	female	64	2	0	IV	Lung	Ad	L858R	First-line	1	PD

*** PS:** ECOG performance status; Ad: adenocarcinoma

2.3 Single cell isolation and DNA extraction

H1975 cell suspension was prepared to isolate single H1975 cell into 96-well plates added with 10ul cell lysis solution in each well (50mmol/L Tris, 1mmol/L EDTA, 0.5% Tween-20, 200mg/L proteinase K) by using flow cytometry (FCM, Becton Dickinson, BD).

For each frozen tissue sample, a 5-μm-thick section was mounted on a membrane slide (Arcturus^{XT}) and then stained with hematoxylin for histomorphologic identification of cells. Dehydration steps were performed: 70% ethanol for 30s, 95% ethanol for 30s, and 100% ethanol for 30s. Then the slides were put in xylene through a 5 min bath to remove Ethanol. After air-dried, the sections were microdissected to capture individual tumor cells incorporating infrared(IR) laser capture systems and ultraviolet(UV) laser cutting systems in LCM(Arcturus^{XT}) instrument according to the standard LCM protocol. In each tumor section 20 - 24 tumor cells were captured. Figure 1 showed the sites where the single tumor cells captured in tumor section roughly.[]In Arcturus^{XT} system, ultraviolet laser cut the wanted single tumor cells, whereas infrared laser melt

thermolabile polymers for cell capture. Following microdissection, we added the cell lysis solution directly to the polymer film and insert the polymer end of the cap into the top of a 500 μL microcentrifuge tube allowing the collection of the whole cell lysates for downstream analysis (Arcturus).[19]

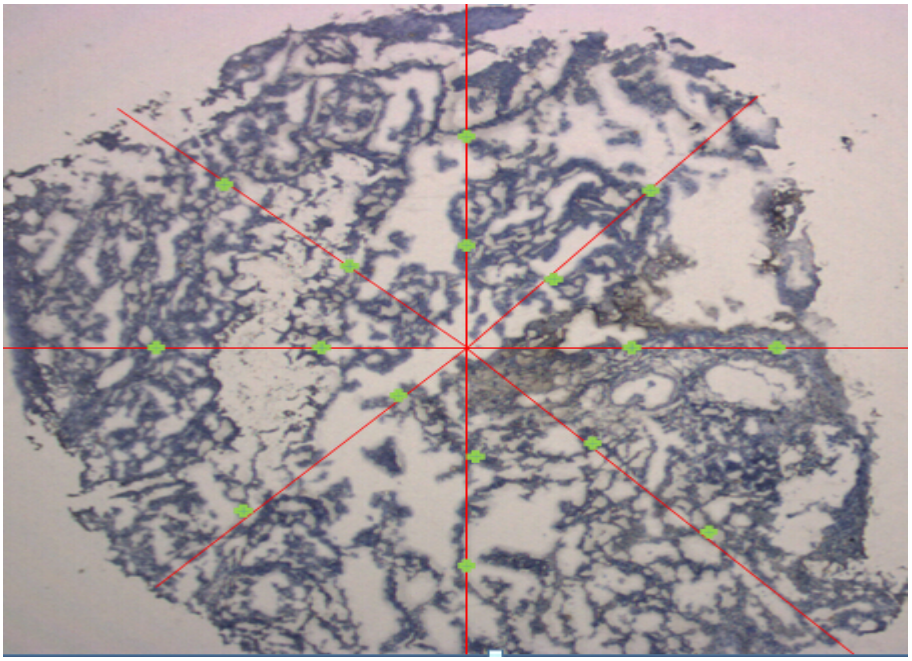


Figure 1. Single tumor cell captured map in one section

(Green crosses represented the sites of single tumor cells captured along the red lines roughly.)

2.4 Single cell PCR analysis

The whole DNA extracted from single cell was submitted to perform nested PCR amplification of *EGFR* 21 exon. The primers and nested-PCR protocol were shown in supplement table 1S[20]. Briefly, for external PCR amplification, the 25ul reaction contained 10ul single cell lysate, 0.5ul primers(Takara) and 1x GoTaq Colorless Master Mix(Promega). For internal PCR amplification, the 25ul reaction consisted of 0.1ul product of external PCR amplification, 10ul RNA-free water, 0.5 internal primers and 1x GoTaq Colorless Master Mix. Then the single-cell nested PCR products were visualized by 2% agarose gel electrophoresis. For H1975 cell line, two blank controls were taken for every six H1975 cells. Beside two blank controls, DNA extracted from cancerous tissue for nested PCR amplification was used as positive control, and two single normal cells captured from each section served as negative controls as well.

Table 1S The nested-PCR amplification primers and protocols for EGFR 21 exon

	primers	Sequence 5'~3'	PCR conditions	PCR product (bp)
External PCR amplification	external forward primer	TCAGAGCCTGGCATGAACATGACCCTG	95°C,5min 95°C, 45sec 61°C, 30sec 72°C, 30sec } 35× 72°C, 7min	297
	external reverse primer	GGTCCCTGGTGTCAGGAAAATGCTGG		
Internal PCR amplification	internal forward primer	CATGAACTACTTGGAGGACCGT	95°C,5min 95°C, 45sec 60°C, 30sec 72°C, 30sec } 35× 72°C, 7min	188
	internal reverse primer	GAAAATGCTGGCTGACCTAAG		

2.5 EGFR mutation analysis by direct sequencing

All nested PCR amplified products based on single cell which showed positive PCR reactions by agarose gel electrophoresis were also sequenced to determine *EGFR* 21 exon status. The products were purified and labeled by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and then sequenced byABI 3100 Genetic Analyzer (Applied Biosystems). Sequence reactions were read and confirmed by two independent experienced readers.

2.6 Statistical analysis

For H1975 cell line, we calculated the efficiency of nested PCR amplification and the rate of allele drop-out(ADO) through direct sequencing.

For tumor samples, the efficiency of nested PCR amplification and the mutational rate in each specimen were computed firstly. Secondly, the nested PCR efficiency and the *EGFR* mutational rate between two groups were also analyzed and compared by X^2 test. α=0.05 (two-sided) as the difference level, and $P < 0.05$ was considered indicative of statistical significance. Statistical analysis was carried out using SPSS 13.0 software (SPSS Inc, USA).

3. Results

3.1 Validation of heterozygous mutation of EGFR in the single cell

A total of 104 individual H1975 cells were isolated. For individual H1975 cells, the efficiency of nested PCR applied to amplify *EGFR* 21 exon was 96.2% (100/104). Supplement figure 1SA showed a picture of agarose gel electrophoresis from nested PCR amplification of single H1975 cells. A total of 93 H1975 cells showed heterozygous mutation of *EGFR* 21 exon. Four cells were homozygous mutation and the remaining 3 H1975 cells were interpreted as *EGFR* wild type, indicating allele drop-out (ADO) occurred during the nested PCR process.

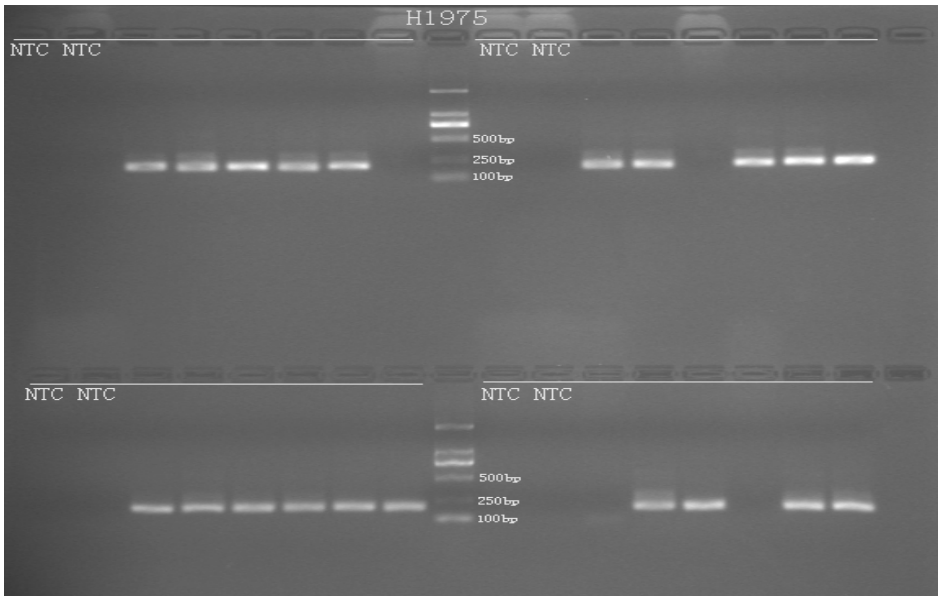
In total, 135 tumor cells and 12 normal cells as negative controls were captured by LCM from the six tumor samples in total (Table 2).

Over all six tumor samples, the total success rate was 88.9% (120/135) by nested PCR. The efficiencies in each tumor sample were listed in Table 2. The efficiency of single-cell nested PCR amplification in Group A was 84.3% (59/70). In Group B the efficiency was 93.8% (61/65). There was not any significant difference about the efficiency of nested PCR between the two groups (X^2=3.119 , P=0.077)(Table 2).

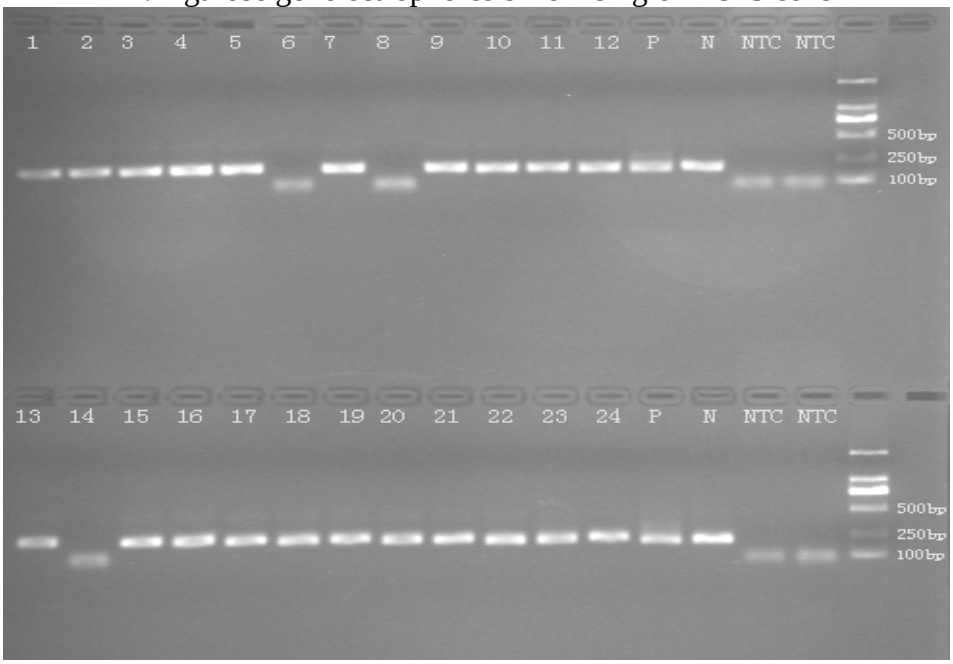
There were no sharp bands detected in all bank controls. For 12 negative controls from single normal cells, 10 normal cells were amplified successfully by nested PCR for *EGFR* 21 exon. A sharp band were detected in all positive controls. An agarose gel electrophoresis picture of single tumor cells from a tissue section was exhibited in supplement figure 1SB.

Table 2. The efficiencies of nested PCR

Patient no.	Number of tumor cells	Nested PCR efficiency (n, %)	P
Group A			
3647	23	19 (82.6%)	
2715	24	21 (87.5%)	
4128	23	19 (82.6%)	
total	70	59(84.3%±2.8%)	
Group B			
3669	20	18(90.0%)	
1813	23	17(91.3%)	0.077
3651	22	22(100.0%)	
Total	65	61(93.8%±5.4%)	



A. Agarose gel electrophoresis from single H1975 cells

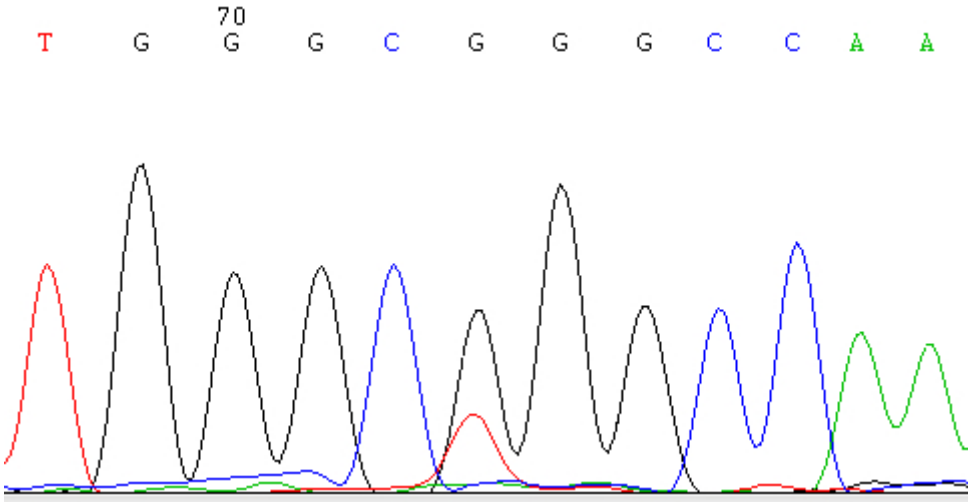


B. Agarose gel electrophoresis from single tumor cells

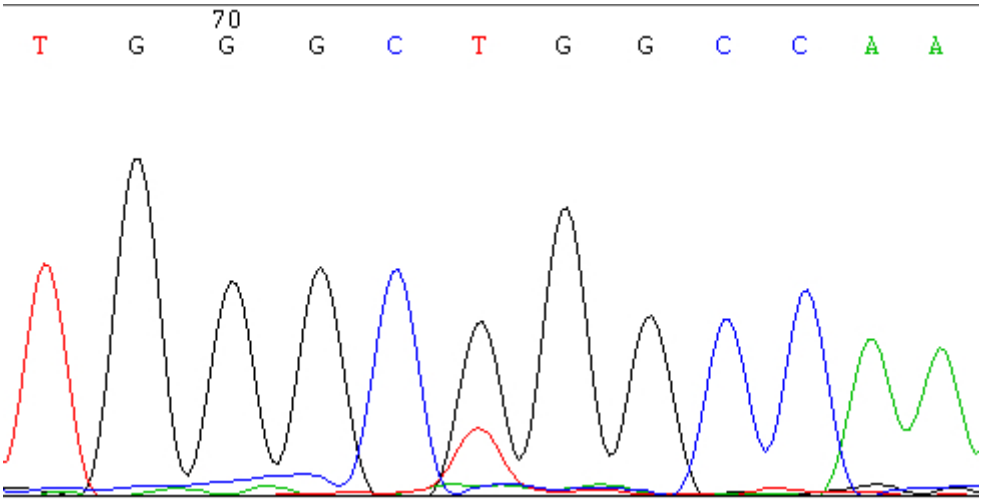
Figure 1S.The example of agarose gel electrophoresis pictures from single-cell nested PCR amplification.

3.2 Results of EGFR detection by direct sequencing

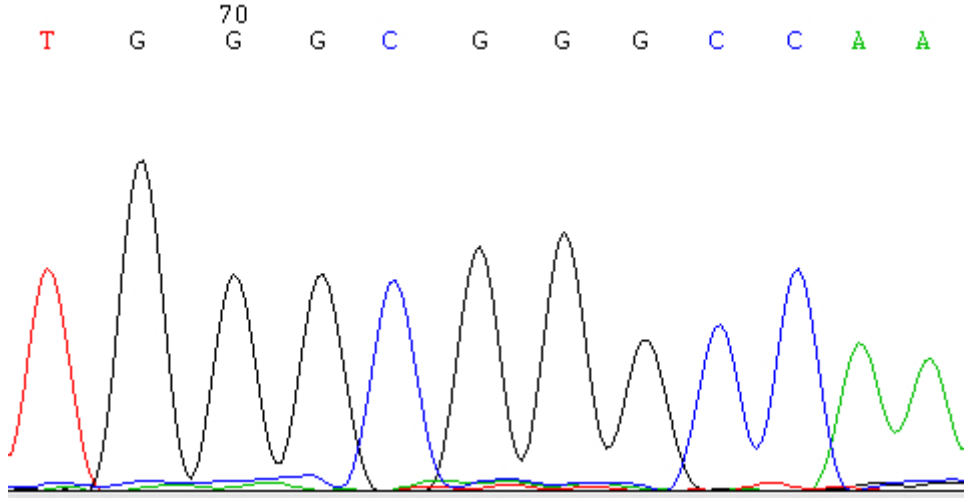
One hundred H1975 cells amplified positive detected by agarose gel electrophoresis were then sequenced to confirm. The success rate was 100 %(100/100). Among them, 93 showed *EGFR* 21 exon L858R heterozygous mutation (Figure 2S A, B). Homogeneous mutation and wild type were detected in 4 cells and 3 cells, respectively. So the rate of allele drop-out (ADO) was 7.0% (7/100) (Figure 2S C, D).



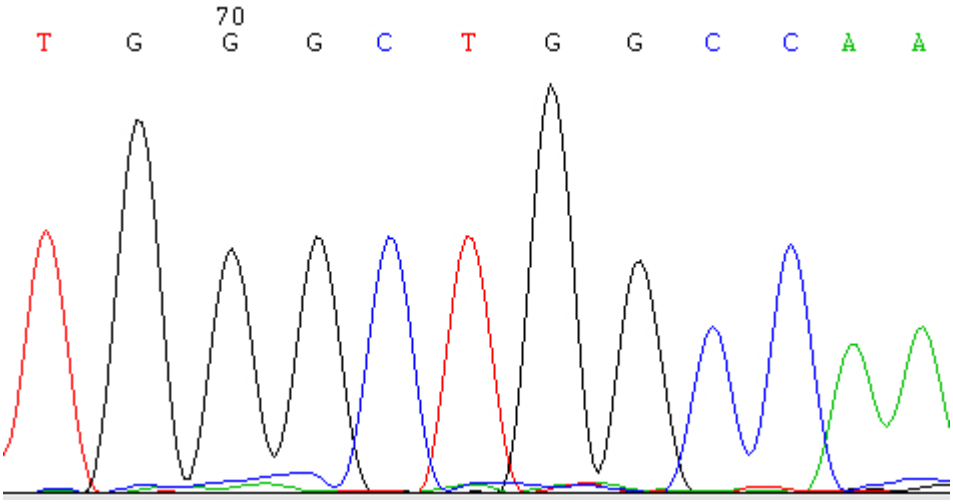
A. One style of heterozygous mutant sequence for single H1975 cell



B. The other style of heterozygous mutant sequence for single H1975 cell



C. Homozygous mutant sequence for single H1975 cell



D. Homozygous wild type sequence for single H1975 cell

Figure 2S. Examples of sequencing picture for EGFR 21 exon from single H1975 cells

Among 120 tumor cells amplified successfully, the rate of *EGFR* 21 exon mutation and wild-type was 77.5 %(93/120) and 22.5%(27/120). The rate of *EGFR* 21 exon mutation were 86.4%±4.9% and 68.9%±2.8% in Group A and Group B, respectively (Table 3). *EGFR* 21 exon mutational rate in Group A was higher than that in Group B(X^2=5.321, *P*=0.021).

Table 3.The rate of EGFR 21 exon mutation in each specimen

Patient no.	Cells number (n)	mutational rate (n,%)	P
Group A			
3647	19	17 (89.5%))	
2715	21	17 (81.0%))	
4128	19	17 (89.5%))	
total	59	51 (86.4%±4.9%)	
Group B			
3669	18	13(72.2%)	
1813	21	14(66.7%)	0.021
3651	22	15(68.4%)	
Total	61	42(68.9%±2.8%)	

4. Discussion

In the study, H1975 cell line results demonstrated the feasibility of *EGFR* detection based on single cells. Furthermore, the single-cell analysis demonstrated intratumoral heterogeneity of *EGFR* activating mutation in lung adenocarcinoma really existed and it might affect the benefit of EGFR-TKI treatment.

In our study, single-cell method described previously was applied to evaluate the heterogeneity of *EGFR* 21 exon L858R mutation in six adenocarcinoma histologically samples. To our knowledge, no similar studies were designed to evaluate intratumoral heterogeneity of *EGFR* activating mutations till now. According to our results, *EGFR* wild-type tumor cells was detected in all the six samples, which suggested the existence of intratumoral heterogeneity for *EGFR* mutation in lung adenocarcinoma. And *EGFR* mutational rate in Group A (PFS>12 months) was higher than that in Group B (PFS<6 months), which indicated that intratumoral heterogeneity of *EGFR* activating mutations might associated with the response to EGFR-TKIs in the treatment of NSCLC patients with *EGFR* activating mutation tumors.

Our results were consistent with our previous study and several other studies[7, 10-12] , but contrary to Yatabe et al. who reported that intratumoral heterogeneity of *EGFR* mutation was rare in lung adenocarcinoma[13]. The authors regarded intratumoral *EGFR* mutational heterogeneity detected as

psedoheterogeneity resulted from mutant allele specific imbalance(MASI) termed by Gazdar' s group[18, 21]. In some areas within a tumor *EGFR* is mutated but not amplified. If there was not any normal cells mixed in these areas, the mutational signal would be equivalent to wild-type signal. But a tumor is always mixed with normal cells which might result in *EGFR* mutated signal diluted and below the threshold of detection. So this situation will lead to pseudoheterogeneous distribution of *EGFR* mutation in lung cancer. However, without mixture of normal cells the psedoheterogeneity did not happen through the single-cell method in our study. Recently Gerlinger et al also demonstrated the presence of marked intratumoral heterogeneity in regard to somatic mutations in driver and passenger genes[22]. In a word, with the limited number of cases studied, we confirmed *EGFR* activating mutation was indeed heterogeneous distribution in lung adenocarcinoma.

In the single-cell analysis, ADO is a stochastic and unique problem to PCR of cellular DNA from one cell and can affect either of the alleles of a given locus or strikes at random. It means only one of the two alleles present in a cell is amplified and detected after PCR and then a heterozygous cell will appear homozygosity[23]. Our results suggested ADO led to 7% error rate in *EGFR* detection for 21 exon within the range from 5% to 15% according to some reports in the field of preimplantation genetic diagnosis (PGD). We also paid attention to contamination of the single-cell method. Blank controls were taken for every 6 single cells tested, and it was encouraging that no non-specifically amplified bands appeared. This demonstrated our procedure for nested PCR amplification did not introduce contaminated DNA. All these results revealed that it was feasible to detect *EGFR* mutation based on single cells, which laid the foundation for *EGFR* detection of scare tumor cells from small specimens.

Present study demonstrated that *EGFR* 21 exon mutational rate in long-term PFS groups was higher than that in short-term PFS groups, which indicating that intratumoral heterogeneity of *EGFR* activating mutation might affect the benefit of EGFR-TKI treatment. During EGFR-TKI treatment, *EGFR* mutant tumor cells were inhibited whereas *EGFR* wild-type tumor cells were trend to proliferation well. Due to this, high *EGFR* mutational heterogeneity in tumors was easy to resistance to EGFR-TKIs. The result was consistent with our previous study that the relative *EGFR* mutation abundance in tumor tissues was associated with benefit from EGFR-TKI treatments. However, because of a few samples in present study, we could not make decision the relationship between intratumoral heterogeneity of *EGFR* activating mutation and benefit of EGFR-TKI treatment in lung adenocarcenoma.

In our study, we selected and detected L858R mutation. Our results indicated that the heterogeneity of L858R have some relationship to the treatment outcome, and to observe the heterogeneity of EGFR, we made a compression between the patients who had longer and shorter PFS. But actually, there was little difference at L858R heterogeneity (86.4%±4.9% vs. 68.9%±2.8%). The reason of such situation may because we did not detect the mutations situation of some genes such as T790M and c-met, which could also affect the PFS. In the future study, we will conduct more comprehensive detection of gene mutation, and to investigate the relationship between mutant heteroplasmy and PFS.

There were still some strength and limitations in our study. In order to reduce the reporting and detection bias, we collected and analyzed the PFS data of patients first , and then detected the heterogeneity of EGFR. Also, there were several limitations. Firstly, only one exon of *EGFR* was detected owing to minute DNA in a single cell. So our study on intratumoral *EGFR* mutational heterogeneity was only focused on 21 exon of *EGFR* activating mutation which also inclues 19 exon deletion. According to some reports with the application of whole genome amplification (WGA) sufficient DNA can be obtained to determine *EGFR* status at multiple sites[20, 24]. Secondly, the issue of ADO was inevitable in the process of single-cell PCR amplification which might lead to false-negative result. Although the frequency of ADO evaluated in our study was low, it should be paid an attention. Fluorescent PCR or digital PCR can be used to reduce the occurrence of ADO[25]. Thirdly, the sample size was small. However, the study aimed to the existence of intratumoral *EGFR* mutational heterogeneity, the relationship between heterogeneity and the therapeutic effect of KTI need large sample validation in the futher suudy. Finally, it was limited for the cases chosen to

study the controversial problem of intratumoral *EGFR* mutational heterogeneity. Despite these disadvantadges, our study had provided evidence for the existence of intratumoral heterogeneity for *EGFR* activating mutation in lung cancer at the level of single cells.

5. Conclusion

In conclusion, we demonstrated it is feasible to perform *EGFR* detection based on single-cell analysis. Then with the application of the method we confirmed the presence of intratumoral *EGFR* activating mutation heterogeneity in lung adenocarcinoma at the single-cell level. Combined with our previous study, present study suggests that intratumoral heterogeneity in the form of the presence, distribution and abundance of *EGFR* activating mutations could contribute to plasma ctDNA mutation load and response to EGFR-targeted therapy.

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