Intratumoral heterogeneity of EGFR-activating mutations in advanced NSCLC patients at the single-cell level

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Abstract

Background: Intratumoral epidermal growth factor receptor (EGFR) mutational heterogeneity is yet controversial in non-small cell lung cancer (NSCLC) patients. Single-cell analysis provides the genetic profile of single cancer cells and an in-depth understanding of the heterogeneity of a tumor.

Methods: Firstly, single H1975 cells harboring the EGFR L858R mutation were submitted to flow cytometry isolation, nested polymerase chain reaction (nested-PCR) amplification, and direct DNA sequencing to assess the feasibility of single-cell direct DNA sequencing. Then, the single cells of patients with lung adenocarcinoma receiving gefitinib were captured by laser capture microdissection and analyzed by the above methods to identify the intratumoral heterogeneity of the EGFR L858R mutant. Three patients with progression-free survival (PFS) > 14 months were categorized as the long PFS group, and 3 patients with PFS < 6 months as the short PFS group. The correlation between the abundance of EGFR L858R mutant and PFS was analyzed.

Results: 104 single H1975 cells were isolated. 100/104 were amplified by nested-PCR and confirmed by direct sequencing. We captured 135 tumor cells from the tissues of six patients. 120 single tumor cells were successfully amplified and sequenced. The rate of EGFR exon 21 mutation was only 77.5% (93/120). Furthermore, the rate of mutation in exon 21 of EGFR was significantly higher in the long PFS group than in the short PFS group (86.4 ± 4.9% vs. 68.9 ± 2.8%, P = 0.021).

Conclusion: Our study suggested the intratumoral heterogeneity of EGFR-activating mutations in lung adenocarcinoma confirmed on the single-cell level, which might be associated with EGFR-TKIs response in lung adenocarcinoma patients harboring the EGFR L858R mutation.

Keywords: Intratumoral heterogeneity, Single-cell analysis, EGFR-activating mutations

Background

Lung cancer is a leading cause of cancer-related deaths all over the world [1]. In recent years, several large randomized controlled clinical trials consistently demonstrated that the tyrosine kinase inhibitors (TKIs) of the epidermal growth factor receptor (EGFR) have a great efficacy in the treatment of patients with non-small cell lung cancer (NSCLC) harboring an EGFR-activating mutation compared to chemotherapy as the first-line treatment [2–10]. The response to EGFR-TKIs is different in patients carrying mutant EGFR. Some patients experienced a progression-free survival (PFS) of > 1 year, whereas others presented a PFS of < 6 months. Recently, intratumoral heterogeneity, intertumoral heterogeneity, and pre- or post-treatment heterogeneity regarding EGFR-activating mutations have been considered as potential causes for the differences in response to EGFR-TKIs [11–14].

Intratumoral heterogeneity arises from the introduction of genetic and epigenetic alterations by genomic and chromosomal instability and different patterns of...
clonal evolution over space and time, and has been reported in various types of cancers, including lung cancer [15, 16]. Nevertheless, studies on the intratumoral heterogeneity of EGFR-activating mutations generated contradicting results [17–19]. These discrepancies might be attributed to the different samples processing methods, including multi-region bulk tissue sampling and manual microdissection analysis. Indeed, these two methods allow the examination on the tissue or multi-cell levels [20]. Consequently, the intrinsic cell-to-cell variation in the EGFR mutation status might be masked by bulk-cell examination and the mutation status of EGFR might be misinterpreted due to the interference from the genetic heterogeneity of the cancer cells. Single-cell analysis directly provides the genetic status of single cancer cells and an in-depth understanding of the genetic characteristics of a tumor by isolating the single cells by flow cytometry (FCM) and laser capture microdissection (LCM) [21]. In addition, single tumor cell analysis might provide a deeper insight into the occurrence of intratumoral heterogeneity of EGFR-activating mutations [22].

In the present study, we investigated the intratumor heterogeneity with single-cell analysis as a definitive approach. Single H1975 cells that harbor the EGFR L858R heterozygous mutation in exon 21 were isolated by FCM and used for evaluating the feasibility of single-cell analysis of the mutation. A previous study by our group demonstrated the presence of EGFR heterogeneity on the tissue level and showed that the relative abundance of EGFR mutation in tumor tissues could predict the benefit of EGFR-TKI treatments [23]. Based on the single-cell method, we explored whether EGFR activating mutation heterogeneity in a tumor did exist in actual lung adenocarcinoma specimens positive for the L858R mutation in exon 21 of EGFR and its relation to EGFR-TKI response.

Methods

Cell culture, single cell isolation, and DNA extraction

The NSCLC cell line H1975, which harbors the L858R heterozygous mutation in exon 21 of the EGFR gene [24], was a kind gift from Professor Tony S. Mok (Prince of Wales Hospital, Hong Kong), and was originally purchased from the American Type Culture Collection (ATCC). The H1975 cells were cultured in RPMI 1640 containing 10% fetal calf serum and incubated at 37 °C in a humidified atmosphere with 5% CO₂. When the cells achieved 80–90% confluence, they were trypsinized to prepare single-cell suspensions that were seeded in 96-well plates and lysed with 10 μL cell lysis solution (50 mmol/L Tris, 1 mmol/L EDTA, 0.5% Tween-20, and 200 mg/L proteinase K). The single cells were isolated using a FASCAtarial II system (BD Biosciences, Franklin Lake, NJ, USA). Before our study, we had conducted a preliminary experiment for single cell isolation with the FASCAtarial II system. H1975 suspension was labelled with Trypan and single cell was sorted by the FCM onto a microscope slide. Then we found the droplet on the slide and confirm whether there was a single cell in it under the microscope (Additional file 1: Figure S1). As previously reported, the rate of successful single cell isolation was >95% and the present study used the optimized parameters (e.g., Ampl:16.4; Drop:1:440; Gap:12; Drop Delay:28.26) from the preliminary experiment [25].

Patients and tissue samples

Patients from the CTONG 0901 clinical trial, treated at the Guangdong Provincial People’s Hospital from 2009 to 2013, were screened (Fig. 1) [26]. The inclusion criteria were: 1) first-line treatment with gefitinib; 2) available PFS data; 3) adequate surgical and frozen specimens of >1 cm in diameter and preserved at the tumor tissue biobank of the Guangdong Lung Cancer Institute (GLCI) [No. GDREC2013185H(R2)]; 4) pure adenocarcinoma; and 5) EGFR L858R mutation in exon 21 by direct sequencing. In order to evaluate the effect of the EGFR mutation on patient prognosis, three patients with PFS >14 months (long PFS) and three patients with PFS <6 months (short PFS) were included (Fig. 1). Tissues from all eligible patients were subjected to LCM and single-cell analysis.

Single cell isolation by LCM and DNA extraction from tissue samples

For each frozen tissue sample, a 5-μm-thick section was mounted on a membrane slide (Arcturus; Thermo Fisher Scientific, Waltham, MA, USA) and stained with hematoxylin for the histomorphological identification of the cells. Dehydration was performed with 70% ethanol for 30 s, 95% ethanol for 30 s, and 100% ethanol for 30 s. Then, the slides were placed in xylene for 5 min. After air-drying, the sections were microdissected to capture individual tumor cells using the LCM system (Arcturus) according to the manufacturer’s protocol. In each section, 20–24 points were selected and one single tumor cell was captured at each point. Figure 2 shows the cell capture map and an example of a section being sampled. According to microscopy, 12 normal cells on the periphery of the tumors were captured as negative controls. In the Arcturus system, the ultraviolet laser cuts the required tumor cells, whereas the infrared laser melts the thermolabile polymers for cell capture. Following microdissection, the cell lysis solution was directly added to the polymer film and placed into a 500-μL microcentrifuge tube [27].
Single-cell nested polymerase chain reaction (nested-PCR) analysis

Whole DNA extracted from a single cell was subjected to nested-PCR amplification of the EGFR 21 exon. The primers and nested-PCR protocol are shown in Table 1 [28]. Briefly, for external PCR amplification, the 25-μL reaction volume contained 10 μL of single cell lysate, 0.5 μL of primers (Takara Bio, Otsu, Japan), and 1× GoTaq Colorless Master Mix (Promega, Madison, WI, USA). For internal PCR amplification, the 25-μL reaction volume consisted of 0.1 μL of the product of the external PCR amplification, 10 μL RNA-free water, 0.5 μL of internal primers, and 1× GoTaq Colorless Master Mix. The single-cell nested PCR products were visualized by 2% agarose gel electrophoresis. Moreover, for the single cell nested-PCR of the H1975 cell line, two blank controls (no cells were isolated) were used for every six H1975 cells. Among the cancer cells captured from the tissues, DNA extracted from cancerous tissues for nested-PCR amplification was used as positive control and that from two normal cells from each section was used as negative controls.

Fig. 1 Study flowchart

Fig. 2 Single tumor cell isolation by laser capture microdissection (LCM). a Single tumor cells capture map. The green crosses represent the sites of single tumor cells captured along six evenly spaced lines radiating from the center of the tumor section (with neighboring sites located approximately 2 mm apart on the lines). In each sample, 20–24 tumor cells were captured (1 cell/site). b, c, d Example of a single cell being sampled from a 5-μm section using the Arcturus system. A target cell (red arrow) was identified on the microdissection system, dissected with an ultraviolet laser, and collected by adhesion onto an infrared laser melted polymer film. b Whole section before sampling. c Whole section after sampling the single cell. d The sampled single cell.
EGFR mutation analysis by direct sequencing
All nested-PCR amplified products based on single cells that showed positive PCR reactions by agarose gel electrophoresis were sequenced to determine the EGFR exon 21 status. The products were purified, labeled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems). The sequence reactions were confirmed by two experienced independent readers (Xuchao Zhang and Zhihong Chen).

Statistical analysis
For the H1975 cell line, the efficiency of nested-PCR amplification and the rate of allele drop-out (ADO) were assessed by direct sequencing. For the tumor specimens, the efficiency of nested-PCR and the mutational rate in each sample were computed first. Subsequently, PCR efficiency and the mutation rate between the two groups were analyzed and compared by the chi-square test. Statistical analysis was carried out using SPSS 21.0 (IBM, Armonk, NY, USA). Two-sided \( P < 0.05 \) was considered statistically significant.

Results
Validation of the heterozygous mutation of EGFR L858R in H1975 cells
Feasibility was tested using single H1975 cells to validate the single-cell analysis of EGFR mutation in NSCLC cells. A total of 104 individual H1975 cells were isolated by FCM. The success rate of nested-PCR for EGFR 21 exon amplification in single H1975 cells was 96.2% (100/104). Figure 3a shows a representative image of agarose gel electrophoresis from nested-PCR amplification of single H1975 cells. All samples positive for EGFR 21 exon amplification yielded sufficient DNA for successful sequencing. The DNA sequencing results showed that the EGFR L858R heterozygous mutation was detected in 93% of the cells (93/100) (Fig. 4a-b). The homozygous mutation and wild-type alleles were detected in four and three cells, respectively, indicating that ADO putatively occurred during the nested-PCR process (Fig. 4c-d). The rate of ADO was 7.0% (7/100).

Intratumoral heterogeneity of EGFR mutation in lung adenocarcinoma tissues
After confirming the validity of the single-cell analysis system, we examined the presence of intratumoral heterogeneity of the EGFR mutation in adenocarcinoma NSCLC tumor tissues. In total, 135 tumor cells and 12 normal cells as negative controls were captured by LCM from the tissue samples obtained from six patients. The EGFR exon 21 was successfully amplified by nested-PCR in 120 tumor cells, including 61 cells from the short PFS group and 59 cells from the long PFS group (Additional file 2: Table S2). A representative agarose gel electrophoresis image of single tumor cells from tissue resections is shown in Fig. 3b. For the 12 negative controls from single normal cells, 10 normal cells were amplified successfully by nested-PCR for EGFR exon 21.

Among the 120 single tumor cells amplified successfully, DNA sequencing showed that the overall rates of EGFR L858R mutation and wild type gene were 77.5% (93/120) and 22.5% (27/120), respectively. None of the six samples presented a 100% mutation rate, thereby confirming the presence of intratumoral heterogeneity.

Association between intratumoral EGFR mutation rate and patient prognosis
To evaluate the effect of intratumoral EGFR L858R mutation rate on the patients’ response to gefitinib therapy, we screened for patients with short (< 6 months) and long (> 14 months) PFS for analysis. The PFS of the long

| Table 1 Nested-PCR amplification primers and protocols for EGFR exon 21 |
|------------------|------------------|------------------|------------------|------------------|
| Primers          | Sequence 5′-3′   | PCR conditions   | PCR product (bp) |
| External PCR amplification | External       | TCAGAGCCTGGCA    | 1) 95 °C, 5 min  | 297              |
|                   | Forward         | TGAACATGACCCCTG  | 2) 35 cycles:   |                  |
|                   | Reverse         | GGTCCCTGGTGTC    | 95 °C, 45 s     |                  |
|                   | Reverse         | AGGAAAATGCTGGG   | 61 °C, 30 s     |                  |
|                   | Reverse         | ATGAACACTTGT     | 5 °C, 5 min     | 188              |
| Internal PCR amplification | Internal       | ATGAACATTGTG     |                  |                  |
|                   | Forward         | GAGGACCGT        | 2) 35 cycles:   |                  |
|                   | Internal        | GAAAATGCTGGCT    |                  |                  |
|                   | Reverse         | GACCTAAG         |                  |                  |

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PFS group were 15, 19, and 22 months, while those of the short PFS group were 1, 3, and 5 months. The baseline characteristics of the six subjects are shown in Additional file 2: Table S1.

The data showed that the mutation abundance of EGFR L858R was higher in the long PFS group than in the short PFS group (86.4 ± 4.9% vs. 68.9 ± 2.8%, \( P = 0.021 \)) and the absolute difference was 17.5% (Table 2).

**Discussion**

Single-cell analysis directly provides the genetic status of single cancer cells and an in-depth understanding of the genetic heterogeneity of the tumor. Therefore, this study evaluated the feasibility of single-cell analysis of EGFR L858R mutation using H1975 cells, to detect the presence of intratumoral heterogeneity in lung adenocarcinoma. The single H1975 cells showed that the EGFR L858R mutation could be reliably detected using single-cell analysis. Herein, we reported for the first time the presence of intratumoral heterogeneity of the EGFR L858R mutation in lung adenocarcinoma on a single-cell level. Indeed, EGFR wild type tumor cells were detected in all six tumor samples. Furthermore, we presented preliminary evidence that the intratumoral EGFR L858R heterogeneity, as measured by the mutation rate, might be associated with the patients’ response to gefitinib therapy.

In the present study, we validated the feasibility of a single-cell analysis system for the detection of EGFR L858R mutation in H1975 cells, using a previously described method [21]. Our study demonstrated a high amplification rate (96.2%) of the EGFR exon 21 by nested-PCR, similar to that described previously [28]. During single-cell analysis, ADO is a stochastic and unique issue pertaining single-cell PCR that can alter the results. In ADO, only one of the two alleles present in a cell is amplified and detected after PCR; therefore, a heterozygous cell will appear homozygous [29]. The present study suggested that ADO led to a 7% error rate in the detection of EGFR mutation in exon 21, which was within the 5–15% range observed in some reports in the field of preimplantation genetic diagnosis (PGD) [29, 30]. In the present study, the contamination of the single cell could also have been an issue. Blank controls were used for every six single cells tested, and non-specific amplification was not detected. This strongly suggests that the procedure for nested-PCR amplification did not introduce any DNA contamination.
According to the results of single-cell analysis, *EGFR* wild type tumor cells were detected in all six tumor samples, which suggested the existence of intratumoral heterogeneity for *EGFR* mutation in lung adenocarcinoma. These results were in agreement with those from previous studies [11, 12, 14, 23]. Nevertheless, Yatabe et al. reported that the intratumoral heterogeneity of *EGFR* mutation was rare in lung adenocarcinoma [17]. The authors regarded the detected intratumoral *EGFR* mutation heterogeneity as pseudo-heterogeneity resulting from the mutant allele-specific imbalance (MASI) [24, 31]. In some areas within a tumor, *EGFR* can be mutated but not amplified. In the absence of the normal cells in these areas, the mutation signal would be equivalent to that of the wild-type; however, a tumor is always mixed with normal cells, which might result in a diluted *EGFR* mutation signal that is below the detection threshold of the method. Therefore, pseudo-heterogeneous distribution of *EGFR* mutation is observed in lung cancer. In this study, the single-cell approach allowed the analysis of tumor cells without the interference of normal cells, and pseudo-heterogeneity was not observed. Recently, Gerlinger et al. also demonstrated the presence of marked intratumoral heterogeneity with respect to somatic mutations in driver and passenger genes [32]. Taken together, these results strongly suggest that the *EGFR*-activating mutation status is heterogeneous in lung adenocarcinoma.

Several large randomized controlled clinical trials (e.g., IPASS [2], WJTOG 3405 [3], OPTIMAL [4], EURTAC [5], LUX-Lung 3 [6], First-SIGNAL [8], NEJ002 [9], LUX-Lung 6 [10]) consistently demonstrated that patients with NSCLC harboring the *EGFR*-activating mutation experienced a 9–13-month median PFS when they received *EGFR*-TKIs treatment. Therefore, we defined PFS > 14 months as long PFS. To evaluate the effect of intratumoral heterogeneity on patient prognosis, we screened for patients with PFS that was representative of short (< 6 months) and long (> 14 months) PFS. The

| Patient no. | Cells number | Mutation rate | P-value |
|-------------|--------------|---------------|---------|
| Long PFS group |              |               |         |
| No.1        | 19           | 17 (89.5%)    | 0.021   |
| No.2        | 21           | 17 (81.0%)    |         |
| No.3        | 19           | 17 (89.5%)    |         |
| Total       | 59           | 51 (86.4%)    |         |
| Short PFS group |            |               |         |
| No.4        | 18           | 13 (72.2%)    |         |
| No.5        | 21           | 14 (66.7%)    |         |
| No.6        | 22           | 15 (68.2%)    |         |
| Total       | 61           | 42 (68.9%)    |         |

Fig. 4 Representative sequencing picture for *EGFR* exon 21 from single H1975 cells. a Heterozygous mutant sequence for single a H1975 cell. b Another heterozygous mutant sequence for single a H1975 cell. c Homozygous mutant sequence for a single H1975 cell. d Homozygous wild type sequence for a single H1975 cell.
present study suggested that the abundance of the EGFR L858R mutation in the long PFS group was higher than in the short PFS group (86.4 ± 4.9% vs. 68.9 ± 2.8%). The results indicated that intratumoral heterogeneity of EGFR-activating mutation might affect the benefits from EGFR-TKIs treatment. During EGFR-TKIs treatment, the EGFR mutant tumor cells are inhibited, whereas the EGFR wild-type tumor cells continue to proliferate. Thus, the high EGFR mutation heterogeneity in tumors could affect the response to EGFR-TKIs. These results are in agreement with those from previous studies [11, 12, 14], including one by our group, which showed that the relative abundance of the EGFR mutation in tumor tissues was associated with the benefits from EGFR-TKIs treatment [23]. Notably, as opposed to the pooled relative abundance measured at the tissue levels by previous studies, single-cell analysis allowed the direct calculation of intratumoral EGFR mutation rates, which might be sensitive indices for patient response to EGFR-TKIs therapy. Nevertheless, the number of patients was quite small and there are many factors influencing the effectiveness of EGFR-TKIs and PFS, including environmental and genetic factors. Therefore, the association between intratumoral heterogeneity of EGFR mutation and response to EGFR-TKIs therapy needs further research.

The PFS was used as the selection criterion for the patients, followed by detection of the heterogeneity of EGFR. Therefore, the reporting and detection bias were small. Nevertheless, the present study has some limitations. Firstly, only one exon of EGFR was tested owing to the small amount of DNA in a single cell. According to the previous reports on the application of whole genome amplification (WGA), sufficient DNA can be obtained to determine the EGFR status at multiple sites [33]. Secondly, the issue of ADO is inevitable in the process of single-cell PCR amplification, which might lead to false-negative results. Although the frequency of ADO was low in the detection of EGFR L858R mutation, it should be considered for the precision of the test; fluorescence PCR or digital PCR can be used to reduce the occurrence of ADO [33]. Finally, the difference between intratumoral heterogeneity and EGFR-TKIs efficacy should be substantiated using large-sized sample studies and other methods like heterozygosity of the mutation, copy numbers, and immunohistochemistry (IHC). Despite these limitations, the present study provides some evidence for the existence of intratumoral heterogeneity of EGFR-activating mutation in lung adenocarcinoma at the level of single cells.

Conclusion
In conclusion, the present study confirmed the intratumoral heterogeneity of EGFR-activating mutation in lung adenocarcinoma by single-cell analysis. The intratumoral heterogeneity of the EGFR L858R mutation might be associated with the gefitinib response in patients with adenocarcinoma NSCLC harboring the mutation. Further studies are needed to explore the potential factors influencing response to EGFR-TKIs in lung adenocarcinoma with EGFR activating mutant.
Competing interests
The authors declare that they have no competing interest.

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